Studies of marine natural products in Tasmania

By

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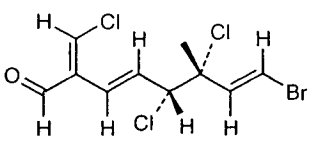
Table of contents

Abstract

Studies of marine natural products in Tasmania

Isolation and structure elucidation of secondary metabolites from two red algae, *Plocamium cartilagineum* and *Laurencia filiformis*, together with the sea hare *Aplysia parvula* are discussed. In addition, chemical relationships between the sea hare *Aplysia parvula* and the red alga *Laurencia filiformis* were also investigated. Some details of studies from an ascidian *Polyandrocarpa lapidosa*, a bryozoan *Watersipora lapidosa*, a bryozoan *Bugula dentata* and its associated nudibranch *Tambje verconis*, as well as a bryozoan *Cribricellina rufa* and its associated pycnogonid *Pseudopallene ambigua* were included. More over, a study of brown algae, *Bellotia eriophorum*, *Sporochnus* species and *Perithalia caudata* was described.

Two new acyclic polyhalogenated monoterpenes, (3*E*,7*E*)-8-bromo-(2*E*)-chloromethylene-(5*R**,6*R**)-dichloro-6-methyloctadien-1-al (1) and (1*Z*,3*E*,7*E*)-8,9-dibromo-(1*Z*,5*R**,6*R**,9)-tetrachloro-6-methyoctatriene (2), as well as two known acyclic polyhalogenated monoterpenes (3-4), were isolated and identified from the red alga *Plocamium cartilagineum*. The structures were established by spectroscopic techniques (^{1}H, ^{13}C NMR, DEPT, HMQC, HMBC, COSY, NOESY experiments, and mass spectrometry).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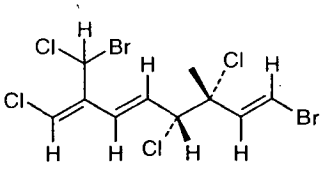
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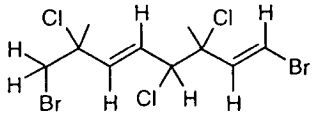
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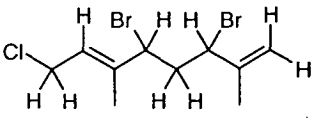
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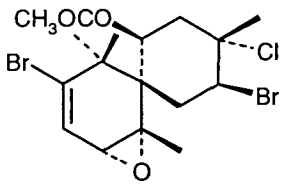
One new chamigrene, 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (5), the known chamigrene, 2,10-dibromo-3-chloro-7-chamigrene (6), deoxyprepacifenol (7), and pacifenol (8) were isolated from both the red alga *Laurencia filiformis* and the sea hare *Aplysia parvula*. On the other hand the known purple pigment, aplysioviolin (9) and the known fimbrolide (10) were isolated only from the sea hare *Aplysia parvula*. The fimbrolide was previously isolated from *Delisea elegans* and *Delisea pulchra* (a synonym for *Delisea fimbriata*). Pentadecanal (11) and its aldol product, namely (*E*)-2-tridecyl-2-heptadec-2-enal (12), were only separated from the

red alga *Laurencia filiformis*. The structure of aplysioviolin has been revised in this study and pentadecanal was separated from the red seaweed *Laurencia* sp. for the first time.



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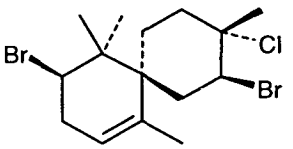

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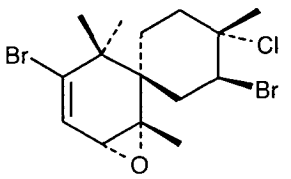

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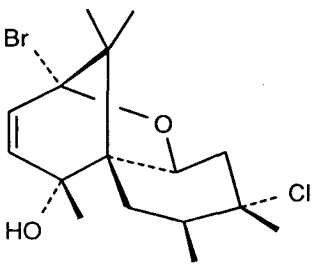

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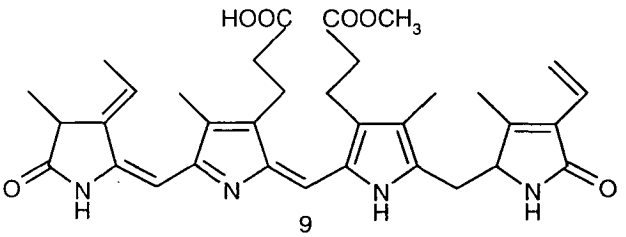

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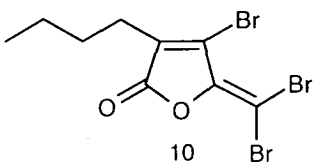

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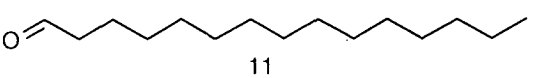

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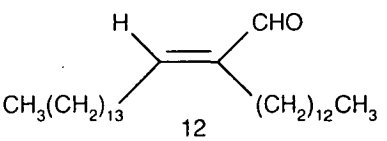

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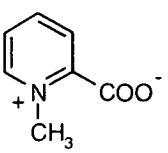
Homarine (13) was isolated from the ascidian *Polyandrocarpa lapidosa*. The structure of homarine was confirmed by synthesis.



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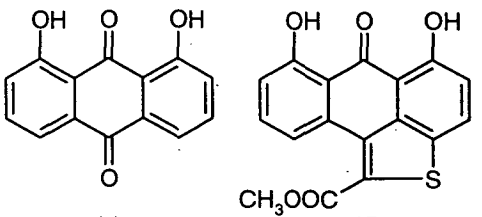
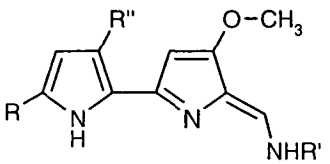
Two known metabolites, 1,8-dihydroxyanthraquinone (14) and 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (15) were separated from the bryozoan *Watersipora subtorquata*.



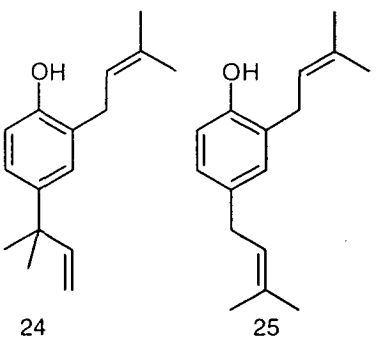
14 15

An investigation of the bryozoan *Bugula dentata* and its associated nudibranch *Tambje verconis* showed that eight known compounds, tamjamines A (16), B (17), C (18), D (19), E (20), G (21), H (22), and J (23) were found from both the bryozoan and the nudibranch.



16 R=R'=R''=H
17 R=Br, R'=R''=H
18 R=H, R'=$CH_2CH(CH_3)_2$, R''=H
19 R=H, R'=$CH_2CH(CH_3)_2$, R''=Br
20 R=H, R'=CH_2CH_3, R''=Br
21 R=Br, R'=CH_2CH_3, R''=H
22 R=Br, R'=$CH_2CH_2CH_3$, R''=H
23 R=Br, R'=$CH_2CH(CH_3)CH_2CH_3$, R''=H

A study of brown algae, *Bellotia eriophorum*, *Sporochnus comosus*, *Sporochnus* species and *Perithalia caudata* showed a variable percentage content of two known compounds (24-25), which were previously isolated from *Perithalia caudata*.



24 25

A preliminary study of secondary metabolites from the bryozoan *Cribricellina rufa* and its associated pycnogonid *Pseudopallene ambigua* revealed that β–carboline metabolites were presented in the bryozoan *Cribricellina rufa* and the pycnogonid *Pseudopallene ambigua* by gas chromatography mass spectrometry.

List of abbreviations

2D NMR	two dimentional nuclear magnetic resonance
BuOH	butanol
CD	circular dichroism
CD_3OD	d_4-deuterated methanol
$CDCl_3$	deuterated chloroform
CI	chemical ionisation
CIMS	chemical ionisation mass spectrometry
COSY	correlation spectroscopy (⟷ COSY)
DCCC	droplet counter current chromatography
DCI	desorption chemical ionization
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethylsulphoxide
DQF-COSY	double-quantum-filtered correlation spectroscopy
ED_{50}	effective dose needed to reduce cell growth by 50%
EI	electron impact
ESI	electrospray ionisation
ESIMS	electrospray ionisation mass spectrometry
EtOAc	ethyl acetate
F	fraction
GCMS	gas chromatography mass spectrometry
gCOSY	gradient correlation spectroscopy
gHMBC	gradient heteronuclear multiple bond coherence
gHMQC	gradient heteronuclear multiple quantum coherence
gNOESY	gradient nuclear Overhauser enhanced spectroscopy
gNOESYps	gradient nuclear Overhauser enhanced spectroscopy phase sensitive
HMBC	heteronuclear multiple bond coherence spectroscopy (⟶ HMBC)
HMQC	heteronuclear multiple quantum coherence spectroscopy(⋯➤ HMQC)
HOHAHA	homonuclear Hartmann-Hahn, one dimensional of TOCSY

HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRFABMS	high resolution fast atom bombardment mass spectrometry
HRLSIMS	high resolution liquid secondary ion mass spectrometry
HSQC	heteronuclear single quantum coherence
ID_{50}	Inhibitory concentration needed to reduce cell growth by 50%
INEPT	insensitive nuclei enhancement by polarization transfer
IR	infrared
J	coupling constant
LC_{50}	lethal concentration needed to reduce cell growth by 50%
LCMS	liquid chromatography mass spectrometry
LSIMS	liquid secondary ion mass spectrometry
MALDI	matrix-assisted laser desorption ionisation mass spectrometry
MeCN	acetonitrile
MeOH	methanol
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MW	molecular weight
ND_3	deuterated ammonia
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser enhanced spectroscopy (◄ · · ► NOESY)
Pet	petroleum ether
PHB	poly(β-hydroxybutyrate)
PTLC	preparative thin layer chromatography
R_f	relative fractional value
RNA	ribonucleic acid
ROESY	rotating frame nuclear Overhauser effect spectroscopy
TLC	thin layer chromatography
TMS	tetramethylsilane
TOCSY	totally correlated spectroscopy
UV-VIS	ultraviolet-visible
δ	chemical shift

List of figures

List of schemes

List of tables

Chapter 1. Marine natural products.

1.1 General introduction.

Natural products are compounds that are intrinsically produced by an organism and known as secondary metabolites. However, they are not typically essential for the organism's survival. Whereas the photosynthetic process and the Krebs cycle products, eg. carbohydrates, proteins, nucleic acid, amino acids, and fats are primary metabolites, which involved in the life processes.[1,2] Secondary metabolites perform such functions as chemical defenses, toxins, antifouling and overgrowth inhibition.[3]

The field of natural product chemistry has been developed as a part of organic chemistry since around the middle of the 19th century. Nowadays the feasibility to explore natural products from the ocean world rather than focusing only on terrestrial organisms is increasing as a result of the possibility to access marine organisms by scuba diving or from deeper water by special submersibles,[4,5] which allow chemists to explore an otherwise untouchable water world. In addition, the problems of taxonomy, marine biology and spectroscopy continue to be diminished because of increasing tools, experts and development of instrumentations.[6,7,8,9] The cooperation of chemists, biologists and taxonomists is essential in order to overcome the overall problems and to help one another solving the problems better.

Chemical ecology, pharmacology, microbiology, biotechnology including genetic engineering, and total synthesis are related to the field of natural products. Some secondary metabolites from both terrestrial and marine sources are of interest. Terrestrial source, eg. paclitaxel or taxol from the bark of the Pacific Yew Tree *Taxus brevifolia*, which are used as drugs and michellamine B from the leaves of the liana *Ancistrocladus korupensis* are potential and useful metabolites. Marine sources, eg. bryostatin-1 from the bryozoan *Bugula neritina*, and dolastatin 10 from the sea hare *Dolabella auricularia* are in the stage of trial drugs and being tested as antimitotic compounds for microtubules.[10,11]

Marine natural products have been annually reviewed since 1984 by Faulkner.[12] The reviews discuss and classify secondary metabolites from the various types of marine organisms. Faulkner also reviewed chemical defenses of marine molluscs such as sea hares of the genus *Aplysia*, ascoglossans, nudibranchs, other opisthobranchs (eg. cephalaspidean

mollusc, notaspidean mollusc), marine pulmonates (eg. onchiids, siphonariids, trimusculiids), limpets, prosobranch molluscs.[13]

Kelecom discussed chemistry of marine natural products from the past through the present and predicted the future trend. During 1987 to 1998, studies on classical algae sources and coelenterates had decreased, sponges had reached a maximum, while echinoderms, molluscs and bryozoans remained the same. In addition, tunicates and microorganisms were increasing in studies of the number of the organisms.[14]

Scheuer reviewed organic chemistry in the ocean, showing some examples of discovering metabolites from various marine organisms such as the dye (tyrian purple) from the marine snail *Murex* spp., the toxin (tetrodotoxin) from the puffer fish *Tetraodon stellatus*, and a skin secretion toxin (9-isocyanopupukeanane) from the nudibranch *Phyllidia varicose*, etc.[15]

McClintock and Baker edited a recent review in marine chemical ecology. This book demonstrated background and introduction to chemical ecology of marine natural products including biosynthesis and as an evolutionary narrative in the first section, an organismal approach to understanding the role of secondary metabolites in mediating trophic interrelationships for the second section, in the next section reviewing cellular and physiological aspects of marine chemical ecology, and applied marine chemical ecology in the last section.[16]

1.2 Thesis aim—marine natural products in Tasmania.

Marine organisms were collected from different locations in Tasmania and screened by using chemical and biological analysis, for example GCMS, TLC, Meyer's test so that several could be selected for further investigation. The aim of this study was to isolate new compounds and elucidate their structures by spectrometric methods. Chemical ecology was included where possible.

More than two hundred and fifty marine organisms were screened in this study. The red algae *Plocamium cartilagineum* and *Laurencia filiformis*; the brown algae *Bellotia eriophorum*, *Sporochnus* species and *Perithalia caudata*; the sea hare *Aplysia parvula*, the nudibranch *Tambja verconis*; the bryozoans *Watersipora subtorquata*, *Bugula dentata* and *Cribricellina rufa*; and the pycnogonid *Pseudopallene ambigua* were selected for detailed examination.

Scuba diving and intertidal collections were used in this work. Extractions with organic solvents, chromatographic methods for isolation and purification techniques and spectroscopic methods together with the brine shrimp bioassay were performed.

1.3 General experimental.

All gas chromatography mass spectrometric analyses (GCMS) were performed on a Hewlett Packard 5890 gas chromatograph coupled to a HP 5790 mass selective detector fitted with an open split interface. A 25 m HP-1 cross-linked polymethylsiloxane capillary column of 0.32 mm internal diameter and 0.52 μm film thickness was used for analysis with 1 μL split injection and 15 *psi* with helium as the carrier gas at a flow rate of 2 mL/min. The GC oven was programmed from 50 to 290 °C at 10 °C/min with a 2 min solvent delay, scanning at the mass range of m/z 40-550. An autosampler HP 7673A was used to inject the sample.

The direct probe mass spectrometry was carried out on a Kratos Concept ISQ mass spectrometer using the direct insertion technique with a source temperature of 200 °C. The electron beam energy was 70 eV for electron impact (EI) spectra and the accelerating voltage was 5.3 KV. Chemical ionization (CI) spectra were acquired using ammonia as the reagent gas. High resolution liquid secondary ion mass spectrometry (HRLSIMS) was performed using *m*-nitrobenzyl alcohol as a standard with resolution of 7000. Peaks are listed in descending order of m/z ratio. Accurate mass measurements were carried out by peak matching at a resolution of 10,000 using perfluorokerosene as a reference. The full scan was recorded at 10 s/decade at 8,000 resolution to give data on all ions in the spectrum.

The electrospray ionization liquid chromatography mass spectrometry (ESI LCMS) experiment was operated on Finnigan MAT LCQ equipped with Waters 2690 Separation Module and Waters 996 Photodiode Array detector using Waters Novapak C18 3.9x150 mm column. A gradient of 80% methanol in water to 95% methanol in water with 0.1% ammonium acetate was used at a flow rate of 0.5 mL/min. for 4 min. then changing to isocratic 100% methanol. The ESI MS/MS was also carried out on Finnigan MAT LCQ and directly injected with a low flow rate of 2 μL/min. Both ESI LCMS and ESI MS/MS were carried out with 4 KV of the spray voltage, 200 °C of capillary temperature and 30 psi.

Fragmentation mechanisms were detected by mass spectrometry/mass spectrometry (MS/MS) using the hybrid option on the concept ISQ. Parent ions were selected by the electric and magnetic sectors, and after collisional activation (100 eV) using argon as the collision gas, their daughters were analysed by the quadrupole sector. To determine parents of a selected daughter ion, the magnetic sector was scanned while transmitting only the required daughter ion through the quadrupole sector.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured at 200 and 400 MHz on Varian Gemini and Varian Inova wide bore spectrometers respectively, using deuterated chloroform or deuterated methanol as solvent with the internal standard tetramethylsilane (TMS). Chemical shifts (δ) are in parts per million (ppm). Coupling constants (*J*) are in Hertz (Hz) and the resonances are indicated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublet (dd), doublet of triplet (dt) and broad (br).

Carbon nuclear magnetic resonance (^{13}C NMR) spectra were measured at 100 MHz on a Varian Inova wide bore spectrometer with a fully decoupled technique. Chemical shifts (δ) are in parts per millions unit (ppm).

Distortionless enhancement by polarization transfer (DEPT) spectra which indicated the degree of protonation of each carbon resonance as a methyl, methylene, and methine group or directly bonded to no hydrogen atom were recorded at 100 MHz on a Varian Inova wide bore spectrometer.

All two dimentional nuclear magnetic resonance (2D NMR) as follows were recorded at 400 MHz on a Varian Inova wide bore spectrometer. Gradient correlation spectroscopy (gCOSY) spectra was used to determine ^{1}H-^{1}H spins that were coupled to each other. Gradient nuclear Overhauser enhanced spectroscopy phase sensitive (gNOESYps) spectra showed which protons were close together in space. Gradient heteronuclear multiple quantum coherence (gHMQC) spectra was used to detect one-bond or directly bonded carbon-hydrogen couplings. Gradient heteronuclear multiple bond coherence (gHMBC) spectra detected correlated long-range proton-carbon couplings.

Melting point was measured on Gallenkamp melting point apparatus. Optical rotations were recorded with a P20 Polarimeter, Bellingham & Stanley Ltd., using dichloromethane or methanol as solvent in 20 cm cell length. Infrared (IR) spectra were measured on a Perkin Elmer FT-IR spectrometer Paragon 1000, using nujol mixed with the

sample and then pasted on sodium chloride cells. Ultraviolet (UV) spectra were recorded in dichloromethane or methanol as solvent on a Shimadzu UV-VIS recording spectrophotometer, using a standard 10 mm path length quartz cell. HPLC was carried out with a Waters 600 multisolvent delivery system connected to a UV Waters 486 tunable absorbance detector. A Dynamax-60A Si gel preparative column was used for compound purification. MPLC was equipped with UA-6 UV/VIS ISCO detector.

Merck Si gels of 70-230 mesh and 230-400 mesh were used for dry column flash chromatography and MPLC respectively. Merck Si gels 70-230 mesh with 60 F_{254} indicator were used for PTLC. Aluminium backed sheets coated with silica 60 F_{254} 0.20 mm thick were used for TLC. Reversed phase C18 silica gel was made by according to the method of Evan *et al.*[17] Sephadex LH-20 of Pharmacia Fine Chemicals was used in the column with the solvent system such as methanol, dichloromethane and methanol (1:1). The Sephadex LH-20 column chromatography was equipped with UA-6 UV/VIS ISCO detector.

The specimens were dried on a Breda Scientific Freeze Dryer JAVAC, model LY-5-FM with condenser capacity 5 litre. The solvents used for specimen extractions and chromatography were fractionated with a Vigreux column.

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Chapter 2. *Plocamium cartilagineum.*

2.1 General introduction and secondary metabolites from *Plocamium*.

The red seaweed genus *Plocamium* is distributed around the world in cool to temperate seas. *Plocamium cartilagineum* (L.) Dixon in this study belongs to Phylum Rhodophyta, class Florideophyceae, order Gigartinales, and family Plocamiaceae. Species of *Plocamium* are normally found on coasts of strong to moderate wave zones. They are quite common as drift on the shore or at depths of 2-26 m.[1,2]

Previous reviews of halogenated monoterpenes from *Plocamium* have appeared in the literature from time to time. In 1975 Fenical[3] reviewed halogenated compounds from the Rhodophyta. Seventeen monoterpenes from red seaweeds were described, six of which were from *Plocamium cartilagineum* and three from *Plocamium* sp. The other eight metabolites, which were acyclic monoterpenes, were from *Desmia hornemanni*. Among the seventeen compounds, the only cyclic monoterpene was from *Plocamium violaceum*.

Crews[4] reported types of halogenated monoterpenes from *Plocamium* in 1977 as 1,5,7-octatriene, 1,5-octadiene, 2,7-octadiene, 1-vinyl-1,5-dimethyl-cyclohexane, 2-vinyl-1,5-dimethyl-cyclohexane and dihydropyran derivatives.

In 1981 Barrow[5] reported the biosynthesis of halogenated cyclic monoterpene metabolites from *Plocamium cartilagineum*, which was carried out through four steps of allylic chlorination with displacement of an oxygen substituent, allylic halogenation, allylic chlorination involving the shift of a double bond and cyclisation.

A critical review of marine monoterpenes was reported by Naylor *et al.*[6] in 1983. No simple correlation of secondary metabolites and organism taxonomy could be concluded.

Since 1984 then there have been annual reviews by Faulkner for marine natural products.[7] In addition, Gribble has also surveyed monoterpenes in naturally occurring organohalogen compounds[8] and reported the diversity of naturally occurring organobromine compounds in 1996.[9]

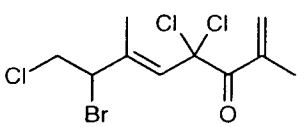
This review will attempt to discuss all monoterpenes and nonterpenoid metabolites discovered in the red seaweed genus *Plocamium* covering the literature published from 1984 to 2000 by SciFinder Scholar searching, focusing on methods of isolation, structure determination and biological activity where possible. SciFinder Scholar is a CAS database,

detailed in http:www.cas.org/SCHOLAR. Secondary metabolites will be discussed in compound types. The drawing of structures is based on the original publications.

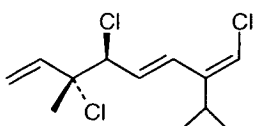
2.1.1 Polyhalogenated acyclic monoterpenes.

In 1984 Stierle *et al.*[10] reported plocamenone (2.1.1.1) from *Plocamium* sp., which was collected off the coast of New South Wales, Australia. The ketone was isolated as the major constituent from hexane extraction and followed by Si gel chromatography. In addition, plocamenone has been shown to be a potent mutagen in the Ames reversion assay.

The same year Crews *et al.*[11] described three new (2.1.1.2, 2.1.2.11-2.1.2.12) and some revised (2.1.1.3, 2.1.2.13-2.1.2.15) monoterpenes from *Plocamium violaceum* and *Plocamium cartilagineum*, which were collected intertidally from Patrick's Point, Humboldt County and Davenport Landing, Santa Cruz County, California, respectively. Acyclic compound (2.1.1.2) was purified by HPLC, 97% hexane/ethyl acetate of fractions 7-9 from gradient elution flash chromatography. Moreover, ^{13}C NMR chemical shifts were used to explain halogen regiochemistry or six-membered ring substituent stereochemistry in marine monoterpenes, together with using new additivity parameters and model compounds in ^{13}C NMR chemical shifts data. However, no incremental constants were available to calculate ^{13}C chemical shifts of tertiary halogenated carbons. It is possible to distinguish between C-Br and C-Cl by ^{13}C NMR since α-substituent effects are large.



2.1.1.1



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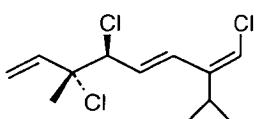
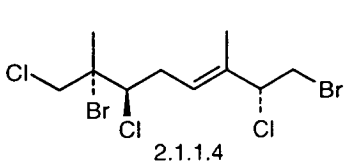


2.1.1.3

In 1985 Blunt *et al.*[12] investigated eight new compounds (2.1.1.4-2.1.1.11) from *Plocamium cartilagineum*. The plants had different appearances and were separated into type I from St. Kilda Rocks and type II from the Old Wharf, Kaikoura, New Zealand. Type I was collected in February, 1979, while type II was collected in Fefruary, 1980. The samples were collected from the two areas, which are geographically close but their constituents were found to be quite different. The Old Wharf sample (type II) contained primarily the C_8 dichlorodienol (2.1.1.12), while the St. Kilda Rocks sample (type I) contained the linear monoterpenes dienes, epoxides and alcohols (2.1.1.4-2.1.1.11, 2.1.1.13) and only a trace of the dichlorodienol (2.1.1.12). It was concluded that the genetic differences occurring between type I and II were the cause of the different metabolites

elaborated by *Plocamium cartilagineum* from different times and from different locations around the Kaikoura Peninsula.

Naylor *et al.*[13] revised the structure of plocamenone (2.1.1.14) from previous structures (2.1.1.15-2.1.1.16)[6] by using new ^{13}C substituent effect values on model compounds.



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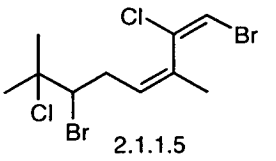

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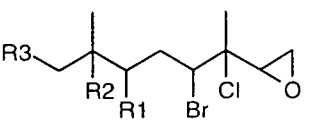
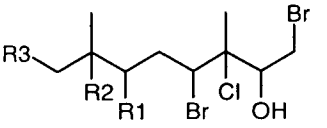
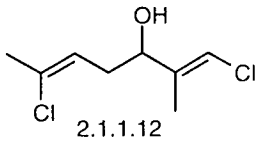




2.1.1.6 R_1=Cl, R_2=Br, R_3=H
2.1.1.7 R_1=Br, R_2=Cl, R_3=H
2.1.1.8 R_1=βCl, R_2=αBr, R_3= Cl

2.1.1.9 R_1=Cl, R_2=Br, R_3=H
2.1.1.10 R_1=Br, R_2=Cl, R_3=H
2.1.1.11 R_1=βCl, R_2=αBr, R_3= Cl



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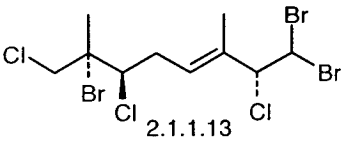
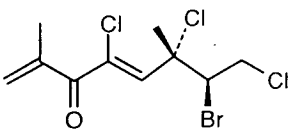


2.1.1.13



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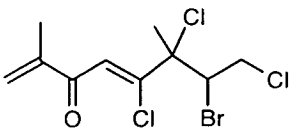

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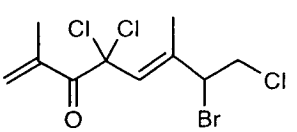
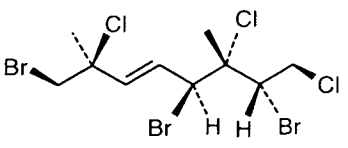


2.1.1.16

Coll *et al.*[14] also reported two new acyclic monoterpenes (2.1.1.17-2.1.1.18) from *Plocamium hamatum*, which was collected from the channel between Orpheus Island and Pelorus Island, Palm Island group in Australia.



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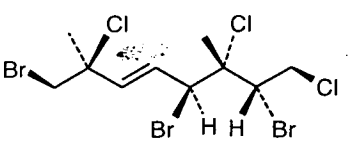
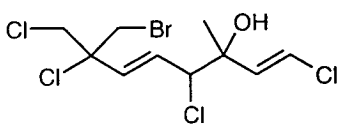


2.1.1.18

Rovirosa *et al.*[15] found one new acyclic (2.1.1.19) and two known (2.1.2.41, 2.1.1.20) halogenated monoterpenes from *Plocamium cartilagineum* which was collected at Rada Covadonga, Antarctic Peninsula. Mass spectrometry of (2.1.1.19) did not display the expected halogen cluster at the molecular ion but the cluster resulting from the loss of an -OH group from the molecular ion was observed. The absolute configuration of 2.1.1.19 was not determined. All three compounds (2.1.1.19-2.1.1.20, 2.1.2.41) showed antibiotic activity against *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus subtilis* and

Staphylococcus aureus. Compound (2.1.1.19) showed the strongest activity against the microorganism *Staphylococcus aureus*. However, all three compounds were inactive against *Staphylococcus epideridis*, *Bacillus arthacis* and *Saccharomyces cerevisiae*.



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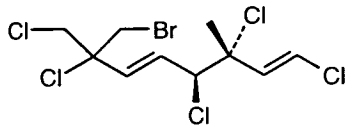
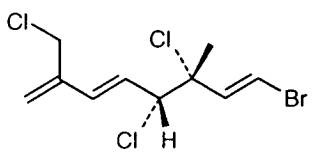


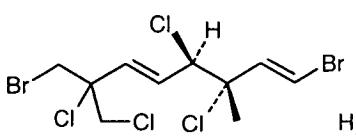
2.1.1.20

König *et al.*[16] reported one new acyclic (2.1.1.21) and three known (2.1.1.22, 2.1.2.39-2.1.2.40) polyhalogenated monoterpenes from *Plocamium cartilagineum* which was collected from northern Spain. HPLC of combined fractions 1 and 2 from vacuum liquid chromatography, using reverse phase C18 with MeOH-MeCN-H_2O 64:9:27 as an eluent, afforded compound 2.1.1.21 as a clear oil. The cyclic monoterpenes (2.1.2.39-2.1.2.40) exhibited potent toxicity towards both *Biomphalaria glabrata* and *Artemia salina*, while the alicyclic compounds (2.1.1.21-2.1.1.22) were not toxic towards these organisms at the concentrations tested. Only relative stereochemistry was assigned for the new metabolite (2.1.1.21).

Polyhalogenated acyclic monoterpene aldehyde (2.1.1.23) was isolated from *Plocamium cartilagineum* from the Portuguese coast.[17] A sequence of Si gel, preparative HPTLC, and reversed phase HPLC techniques was used for purification of compound (2.1.1.23). The structure was determined using the basic NMR, DQF-COSY, ^{1}H-^{1}H COSY and MS, however, only the relative stereochemistry was addressed. The hexane extract containing (2.1.1.23) was lethal for the fish *Lesbites reticulatus* in one hour at concentrations of less than 50 mg/L.



2.1.1.21



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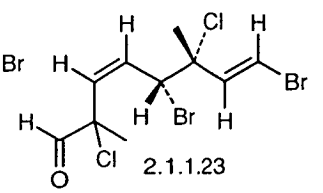
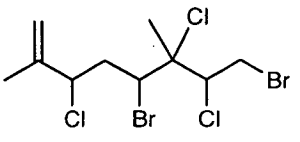


2.1.1.23

In 1999 König *et al.*[18] also investigated one new (2.1.1.24) and three known (2.1.1.25-2.1.1.27) acyclic halogenated monoterpenes from *Plocamium costatum* which was collected from Deep Glen Bay, Eaglehawk Neck, Tasmania, Australia at a depth of 25-30 m. The dichloromethane extract and compound (2.1.1.26) deterred settlement of barnacle larvae, suggesting a potential ecological role. All (2.1.1.24-2.1.1.27) showed little

or no effect on antimicrobial and antialgal activities, and none demonstrated HIV-1 RT inhibitory activity. Compound (2.1.1.27) had only weak effects and only toward brine shrimp at a test concentration of 0.5 mg/mL with *Artemia salina* and *Caenorhabditis elegans*.



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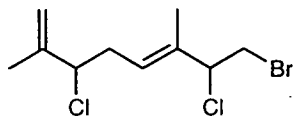

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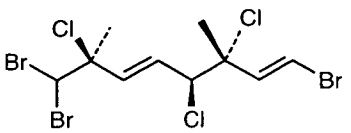

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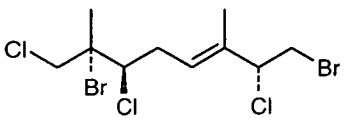
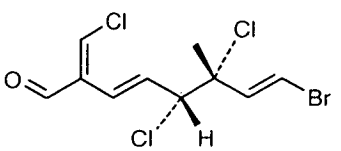


2.1.1.27

In 2000, two new (2.1.1.28-2.1.1.29) and two known (2.1.1.30-2.1.1.31) polyhalogenated acyclic monoterpenes were isolated from *Plocamium cartilagineum*, which was collected from the East coast of Tasmania, Australia.[19] The fraction containing halogenated monoterpenes showed one hundred percent mortality at concentrations of 92.5 μg/mL or greater after 15 hours with *Artemia salina*. These results are also presented in this thesis.



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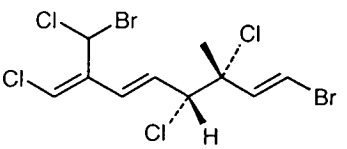

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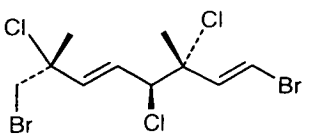

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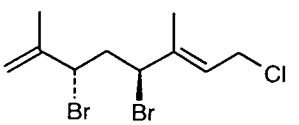
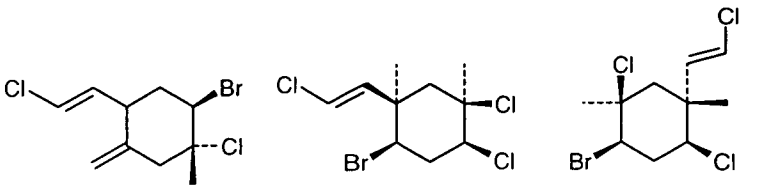


2.1.1.31

2.1.2 Polyhalogenated cyclic monoterpenes.

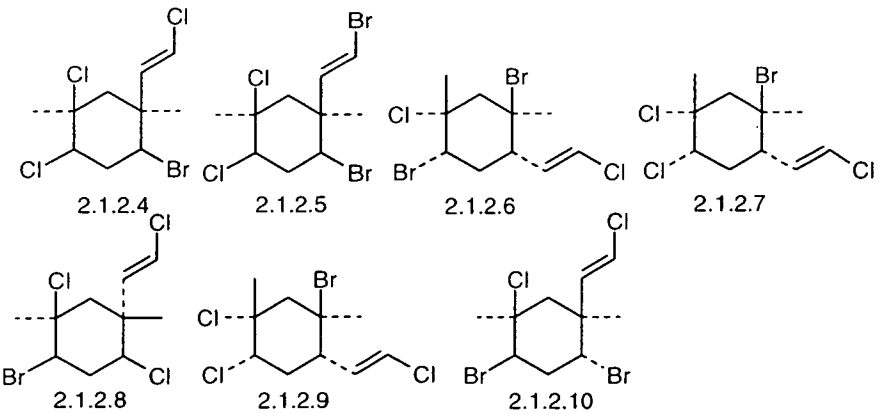
Capon *et al.*[20] reported two compounds isolated from the red seaweeds *Plocamium mertensii* and unclassified *Plocamium* sp., which were collected off Carnac Island and from the drift on Rottnest Island, Western Australia, respectively. A new compound, (1*R*, 2*S*, 4*S*, 1'*E*)-2-bromo-1-chloro-4-(2'-chloroethenyl)-1-methyl-5-methylenecyclohexane (2.1.2.1) was purified by sublimation from a dichloromethane soluble portion and combined with recrystallization from methanol/water of rapid silica filtration to give an identical compound to the sublimed one, mp. 46.5-47.5 °C. Whereas the unidentified *Plocamium*

species yielded (1*R*, 2*S*, 4*R*, 5*R*, 1'*E*)-4-bromo-1,2-dichloro-5-(2'-chloroethenyl)-1,5-dimethylcyclohexane (2.1.2.2), which was purified through Sephadex LH-20 and recrystallized from hexane as white needles, mp. 105.5-106 °C. The close correspondence of the ^{13}C NMR data of (2.1.2.2) with that previously reported for (2.1.2.3) strongly suggested these two compounds were identical. Although no comparison with the authentic mertensene sample in this paper was done, the corrected structure of mertensene was confirmed to be (2.1.2.2), not (2.1.2.3) by X-ray crystallography.

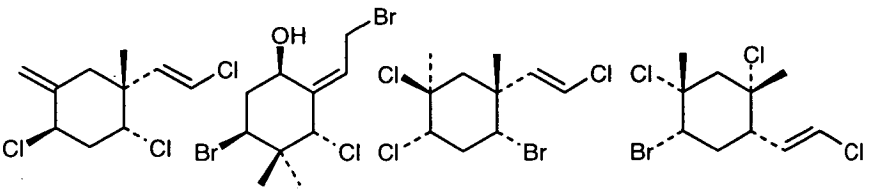


2.1.2.1 2.1.2.2 2.1.2.3

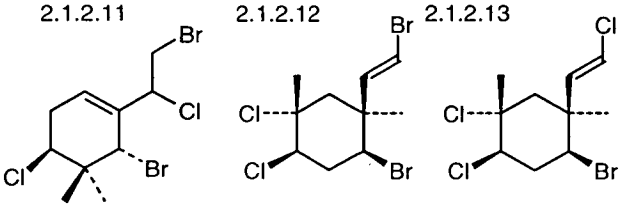
Castedo *et al.*[21] found one new (2.1.2.4) and six known (2.1.2.5-2.1.2.10) compounds from *Plocamium coccineum* Lyngb., which was collected intertidally at Bastiagueiro, Aguino, La Lanzada and Patos, and by dredging at Ria de Arosa from the coast of north-west Spain between April and October. The different batches were shown by HPLC to contain the same composition of compounds (2.1.2.4-2.1.2.10). Coccinene (2.1.2.4) was separated as a crystalline solid, mp. 65 °C from reverse phase HPLC.



In 1984 Crews *et al.*[11] also reported two new (2.1.2.11-2.1.2.12) and some structurally revised (2.1.1.13-2.1.1.15) cyclic monoterpenes from *Plocamium cartilagineum* and *Plocamium violaceum* in California. In 1985 Sardina *et al.*[22] revised two structures (2.1.2.16-2.1.2.17) from *Plocamium coccineum* by using 2D NMR and NOE difference spectroscopy. It was considered that crystals of the previous assigned structures were damaged by X-ray irradiation.

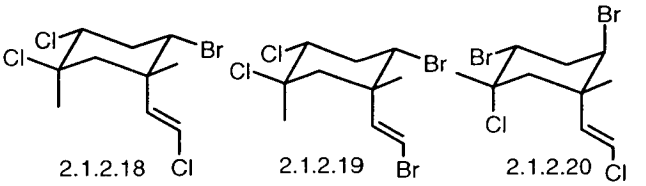


2.1.2.11 2.1.2.12 2.1.2.13 2.1.2.14

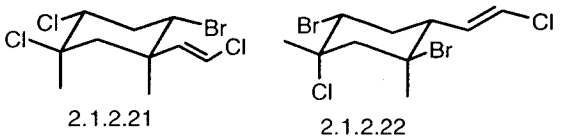


2.1.2.15 2.1.2.16 2.1.2.17

In 1986 Sardina *et al.*[23] also discussed ^{13}C NMR chemical shifts in the stereochemistry of cyclic monoterpenes (2.1.2.18-2.1.2.22) from *Plocamium coccineum*. The calculated minimum energy conformers of the compounds agreed with the geometries deduced experimentally by using a Karplus equation. Using 2D NMR and NOE difference spectroscopy and molecular mechanics calculations led to a complete structural analysis.

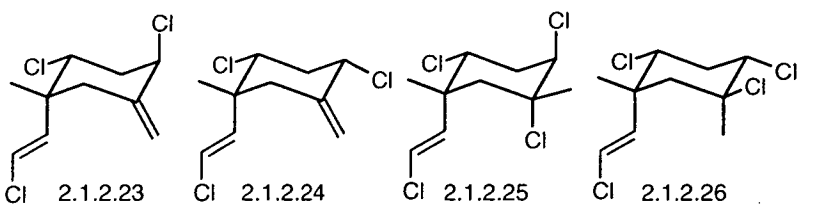


2.1.2.18 2.1.2.19 2.1.2.20

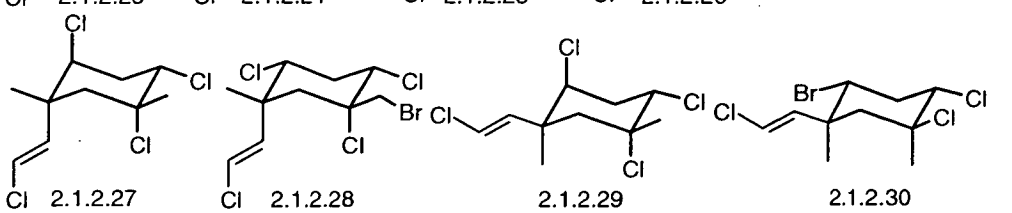


2.1.2.21 2.1.2.22

San-Martin *et al.*[24] reported variations in composition of a number of known cyclic monoterpenes (2.1.2.23-2.1.2.30) of *Plocamium cartilagineum* from the Chilean Coast by GC analysis. Neither ^{1}H NMR nor GC on the semipurified oils could detect acyclic compounds.

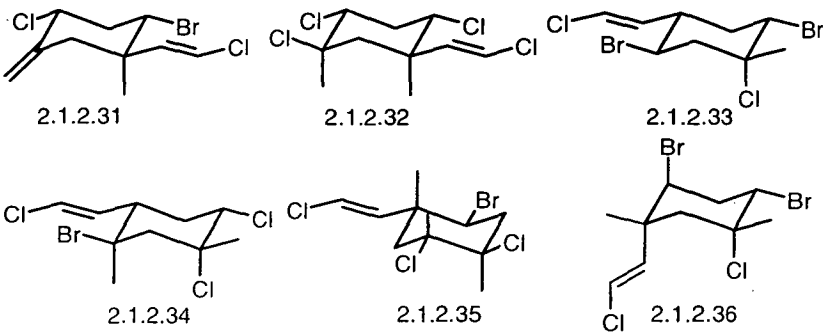


2.1.2.23 2.1.2.24 2.1.2.25 2.1.2.26



2.1.2.27 2.1.2.28 2.1.2.29 2.1.2.30

Coll *et al.*[14] reported three new halogenated monoterpenes (one cyclic, (2.1.2.31) and two acyclic monoterpenes, (2.1.1.22-2.1.1.23)) and a revised mertensene (2.1.2.32) from *Plocamium hamatum*, which was collected from the channel between Orpheus Island and Pelorus Island, Palm Island group in Australia. The structures were confirmed by X-ray crystallography.



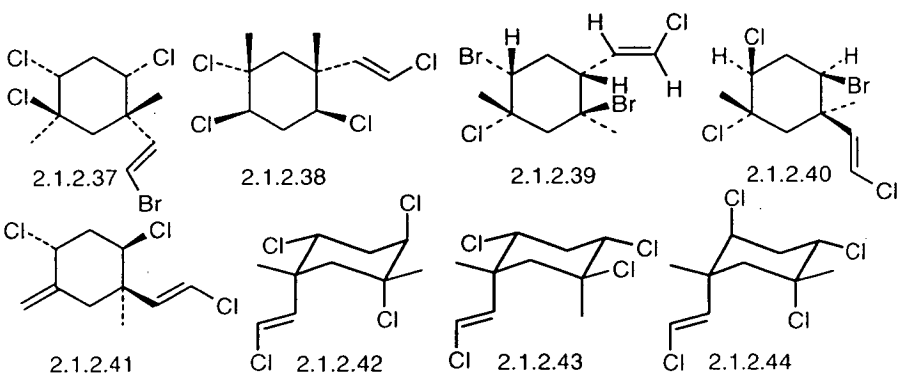
Quinoa *et al.*[25] reported a mixture of cyclic polyhalogenated monoterpenes (2.1.2.18-2.1.2.19, 2.1.2.33-2.1.2.36) from the digestive gland of *Aplysia punctata* but no halogenated compounds were found in *Aplysia depilans*. *Aplysia punctata* were collected at lowtide at Agnino (La Coruna) and Patos (Pontevedra). *Aplysia depilans* were collected by diving at La Coruna and Villagarcia de Arosa. In order to find the origin of these compounds, some red algae were investigated. The red algae *Plocamium coccineum*, *Laurencia pinnatifida*, *Ceramium ciliatum*, *Calliblepharis lanceolata*, *Plumaria elegans*, *Corallina officinalis*, *Gracillaria verucosa*, *Porphyra laciniata* and *Lomentaria articulata* were collected at low tide on the coast of La Coruna and Pontevedra. The HPLC and GLC traces of *Plocamium coccineum* extracts were almost identical with those of the mixture obtained from *Aplysia punctata*, but no halogen-containing compounds were found in the other red seaweeds. In addition, the crude mixture of (2.1.2.18-2.1.2.19, 2.1.2.33-2.1.2.36) was toxic to the larvae of the crustacean *Artemia salina*, with a LC_{50} of 1 μg/0.5 mL.

Telfairine (2.1.2.37) was isolated from the red alga *Plocamium telfairiae* which was collected at Wakasa Bay, Fukui Prefecture, Japan.[26] Its structure was elucidated by ^{1}H, ^{13}C NMR, MS and NOE difference spectrum analysis. This compound exhibited 100% insecticidal activities against mosquito larvae, *Culex pipiens pallens* at 10 ppm.

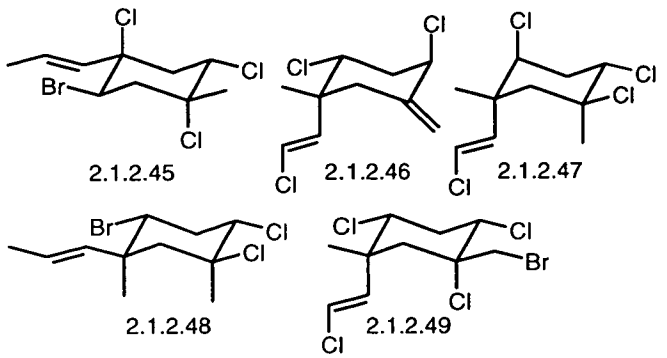
Watanabe *et al.*[27] also isolated two insecticidal polyhalogenated monoterpenes, telfairine (2.1.2.37) and aplysiaterpenoid A (2.1.2.38) from *Plocamium telfairiae*, which was collected at Wakasa Bay, Fukui Prefecture, Japan. Telfairine (2.1.2.37) and

aplysiaterpenoid A (2.1.2.38) showed strong insecticidal activities as a 80 and 60 percent mortality against the German cockroach *Blatella germanica*, respectively. LC_{50} values of telfairine (2.1.2.37) against the susceptible strains of mosquito larvae *Anopheles gambiae* was 1.1 ppm and could not be calculated for the dieldrin-resistant strains of mosquito larvae *Anopheles gambiae* since the mortalities at 1.25 and 2.5 ppm were 0% and 100%, respectively. While LC_{50} values of aplysiaterpenoid A (2.1.2.38) against the susceptible and the dieldrin-resistant strains of mosquito larvae *Anopheles gambiae* were 0.1 and 0.24 ppm, respectively.

In 1990 König *et al.*[16] also reported two known cyclic monoterpenes (2.1.2.39-2.1.2.40) and one new acyclic (2.1.1.21) monoterpene from *Plocamium cartilagineum* in Spain. Rovirosa *et al.*[15] also reported a known cyclic monoterpene (2.1.2.41) from *Plocamium cartilagineum* from the Antarctic Peninsula. San-Martin *et al.*[28] found four new alicyclic monoterpenes (2.1.2.42-2.1.2.45) from *Plocamium cartilagineum* from the Chilean coast, together with four known cyclic monoterpenes (2.1.2.46-2.1.2.49).

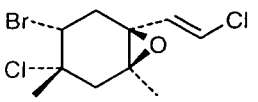


In 1991 Sakata *et al.*[29] also isolated aplysiaterpenoid A (2.1.2.38) from the red alga *Plocamium leptophyllum*, which was collected at Toyama Bay, Japan. This compound, which was previously isolated from the red alga *Plocamium telfairiae* and the sea hare *Aplysia kurodai*, showed feeding inhibitory activity at a level of 40 μg against the marine herbivores, the abalone *Haliotis discus*, the gastropod *Turbo cornutus*, the top shell *Omphalius pfeifferi*, and the sea urchin *Strongylocentrotus intermedius*.



2.1.3 Polyhalogenated epoxymonoterpenes

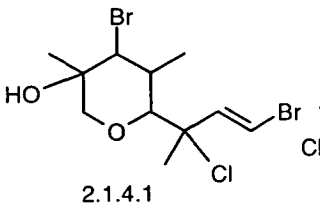
Abreu *et al.*[30] investigated the $CHCl_3$ extract of *Plocamium cartilagineum* collected in Sesimbra, Portugal which was fractionated by Si gel flash chromatography using hexane with increasing proportions of EtOAc to give 4-bromo-5-chloro-2-(*E*)-chlorovinyl-1,5-dimethyl-1,2-epoxycyclohexane (2.1.3.1), mp. 49-50 °C. Mass spectrometry, basic NMR, ^{13}C-INEPT, NOE, and COSY experiments were used for the structure elucidation and a halogen regiochemistry was assigned by comparison of its ^{13}C NMR data with those of model compounds.



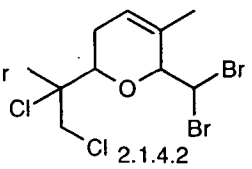
2.1.3.1

2.1.4 Pyran halogenated monoterpenes

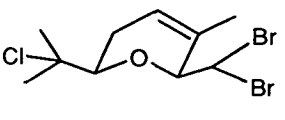
Cueto *et al.*[31] reported four new tetrahydropyran monoterpenes, plocamiopyranoid (2.1.4.1) and compound (2.1.4.2) from *Plocamium cartilagineum* and *Pantoneurines plocamioides*. Pantoneurines A (2.1.4.3) and B (2.1.4.4) from *Pantoneurines plocamioides*, were collected off the Chilean coast. Structures were determined by using COSY, HMQC, HMBC, NOE and PCMODEL. In addition, 2D ROESY NMR confirmed the structure of (2.1.4.1). The interesting structural relationship of these compounds from *Plocamium cartilagineum* and *Pantoneurines plocamioides*, which belong to different orders (Gigartinales and Ceramiales, respectively), suggested a requirement of taxonomic revision.



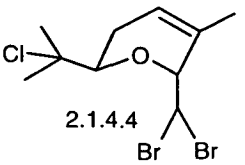
2.1.4.1



2.1.4.2



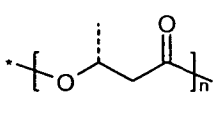
2.1.4.3



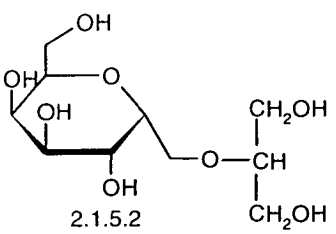
2.1.4.4

2.1.5 Nonterpenoid compounds

Poly(β-hydroxybutyrate) (PHB) (2.1.5.1) and 2-O-α-D-galactopyranosyl-glycerol (fluridoside) (2.1.5.2) were isolated from the red alga *Plocamium cartilagineum*, which was collected from Figueira da Foz, on the coast of Portugal by Abreu *et al.* in 1997.[32] The red alga *P. cartilagineum* was extracted with hexane, chloroform and ethanol. Repeated treatment of the chloroform extract with hexane and acetone gave five fractions of a colourless crystalline solid PHB, mp. 175-176 °C. This was the first report of PHB occurrence in a macroalga. Reversed phase flash chromatography of the ethanol extract using H_2O with increasing proportions of MeOH as eluent, afforded the new secondary metabolite (2.1.5.2) as a water-soluble colourless solid, mp. 120-122 °C. Floridoside is the main low molecular carbohydrate occurring in many red algae and this was the first time that it had been reported in *Plocamium cartilagineum*. Both structures were established on the basis of NMR experiments. In addition, the structure of floridoside was confirmed by GCMS analysis of the permethylated derivatives and the hexaacetate resulting from acid hydrolysis of floridoside followed by $NaBH_4$ reduction and peracetylation.



2.1.5.1



2.1.5.2

2.2 Results and Discussion.

Plocamium cartilagineum was collected at Mayfield Bay by scuba diving and gathered on Schouten Beach after a storm in the area. Schouten Beach is approximately 14 km NNE from Mayfield Bay and both are part of the western side of Great Oyster Bay, east coast Tasmania. Freeze-dried samples of both collections were kept separate and extracted with dichloromethane and methanol. Further purification by dry-column flash Si gel chromatography, PTLC, MPLC and HPLC afforded two new metabolites (3*E*, 7*E*)-8-

bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28) and (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29) together with two known acyclic polyhalogenated monoterpenes (2.1.1.30-2.1.1.31) from both collections. The compound (2.1.1.28) was obtained as an oil, 32 mg (equivalent to 0.006% of dry wt) while the compound (2.1.1.29) was obtained as an oil, 637 mg (equivalent to 0.117% of dry wt). The structures were investigated by spectroscopic techniques.

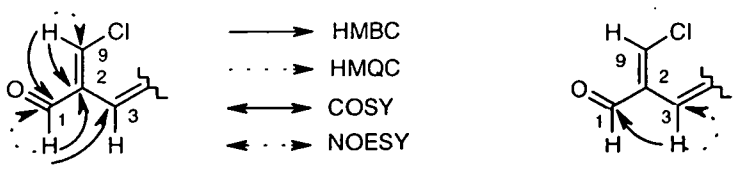
2.2.1 Structure of (3*E*, 7*E*)-8-bromo-(2*E*)-chloromethylene-(5*R**, 6*R**)-dichloro-6-methyloctadiene-1-al (2.1.1.28).

The CIMS gave $C_{10}H_{14}BrCl_3NO$ $[M+NH_4^+]$, which indicated a molecular formula of $C_{10}H_{10}BrCl_3O$, having four degrees of unsaturation. The mass spectrum displayed a molecular ion cluster of $BrCl_3$ species with the value of 347.93243 (52.1), 349.93003 (100), 351.92739 (65.0), 353.92471 (18.3), 355.92220 (2.5).

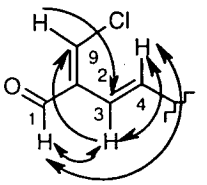
The ^1H NMR spectrum (Figure 2.2.1.1, Table 2.2.1.1, $CDCl_3$ as solvent) showed an aldehyde proton (H-1) at 9.55 ppm with a corresponding methine carbon (C-1) at 189.3 ppm in the ^{13}C spectrum, as well as carbonyl stretching of aldehyde at 1726 cm^{-1} in the IR spectrum. The connection between C-1 and H-1 was confirmed by HMQC.

The ^{13}C NMR spectrum (Figure 2.2.1.2, Table 2.2.1.1) showed ten carbons; the DEPT spectrum displayed one methyl carbon at 28.0 ppm, and seven methines (189.3, 144.3, 137.4, 136.9, 133.7, 123.0, 110.7, 71.6, and 69.5). Therefore, two quaternary carbons were at 137.4 and 71.6 ppm.

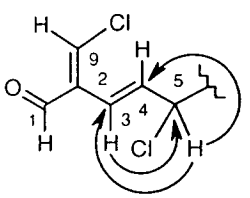
HMBC established connectivities of the aldehyde carbon (C-1) to a methine proton (H-9) at 7.15 ppm, which was also linked to a quaternary carbon (C-2) at 137.4 ppm. Likewise, the quaternary olefin (C-2) was connected to the aldehyde proton (H-1) and then the aldehyde proton (H-1) was linked to a methine carbon (C-3) at 123.0 ppm by HMBC. HMQC confirmed a connection of a methine olefin (C-9) at 144.3 ppm and H-9 at 7.15 ppm. The low field chemical shifts implied an attached chlorine atom. [6, 17, 33] Moreover, the aldehyde carbon (C-1) was connected to a methine proton (H-3) at 6.55 ppm by HMBC. HMQC revealed a connection of H-3 and C-3.

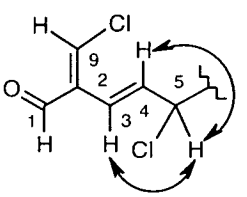


Similarly, C-3 at 123.0 ppm was linked to H-9 at 7.15 ppm and H-3 at 6.55 ppm was connected to C-9 at 144.3 ppm by HMBC. HMQC displayed a connection of H-4 at 7.01 ppm to C-4 at 133.7 ppm. COSY correlations between H-3 to H-4, H-1 to H-3 and H-1 to H-4 were shown. The coupling constant ($J_{3,4}$ 15.9 Hz) of H-3 and H-4 supported a *trans* geometry for the double bond.

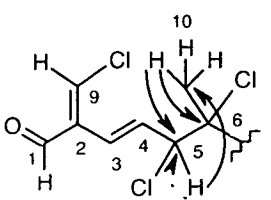


Connectivities by HMBC between H-3 to a methine saturated carbon (C-5) at 69.5 ppm, which implied an attached chlorine atom,[6, 17, 33] C-3 to H-5 at 4.54 ppm, and H-5 to H-4 were detected. Empirically derived substituent effects in linear and branched alkanes demonstrated a small but consistent carbon chemical shift difference for chlorine and bromine. Primary chlorines resonate approximately 11 ppm downfield from the corresponding bromine carbon. Likewise, secondary carbons bearing chlorine resonate approximately 7 ppm toward lower field than the corresponding brominated carbon.[6] COSY correlations between H-4 to H-5 and H-3 to H-5 were displayed. The coupling constant ($J_{4,5}$ 8.9 Hz) of H-4 and H-5 was also revealed.



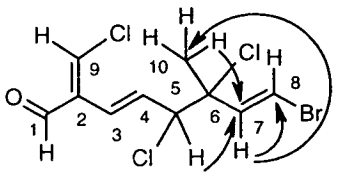


HMBC displayed connectivities of C-5 to H-10 at 1.82 ppm, H-5 to C-10 at 28.0 ppm, and $(H\text{-}10)_3$ to a saturated quaternary carbon (C-6) at 71.6 ppm. The low field chemical shift at C-6 implied an attached chlorine atom.[6, 16, 17, 18, 33, 34] HMQC showed linkages of C-5 to H-5 and C-10 to H-10.

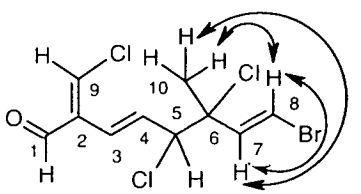


An olefinic methine carbon (C-7) at 136.9 ppm was connected to H-5 and to methyl protons $(H\text{-}10)_3$ by HMBC. Likewise, an olefinic methine proton (H-7) at 6.46 ppm was

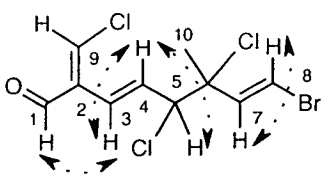
linked to C-10 and to another olefinic methine carbon (C-8) at 110.7 ppm by HMBC. The downfield chemical shift of C-8 implied an attached bromine atom. [6, 17, 33] A coupling constant ($J_{7,8}$ 13.5 Hz) of H-7 and H-8 indicated a *trans* geometry. HMQC showed connections of H-7 to C-7 and H-8 to C-8.



In addition, COSY showed correlations between H-7 and H-8, H-7 and $(H\text{-}10)_3$, and $(H\text{-}10)_3$ and H-8.



The aldehyde proton (H-1, δ 9.55 d, $J_{1,3}$ 2.0 Hz) was coupled to the vinylic proton (H-3 at 6.55 ppm) by gCOSY (Figure 2.2.1.4) and gNOESY (Figure 2.2.1.5). The allylic proton (H-5, δ 4.54 d, $J_{5,4}$ 8.8 Hz) was coupled to the vinyl proton (H-4) and showed a long range coupling to the vinylic proton (H-3, $J_{5,3}$ 0.8 Hz). [34] A gNOESY experiment revealed interactions between H-1 and H-3, H-3 and H-4, H-4 and H-5, and H-7 and H-8 as shown. The coupling constants ($J_{3,4}$ 15.9 and $J_{7,8}$ 13.5 Hz) supported the *trans* geometry for the double bonds (between H-3 and H-4, and H-7 and H-8).



The base peak at m/z 167, 169, 171 showing BrCl cluster ions was observed in the mass spectrum (a proposed MS fragment as Scheme 2.2.1.1) to support the existence of a structure moiety of fragment A. The fragmentation pattern observed in the mass spectrum of compound 2.1.1.28 is consistent with the proposed structure (scheme 2.2.1.1).

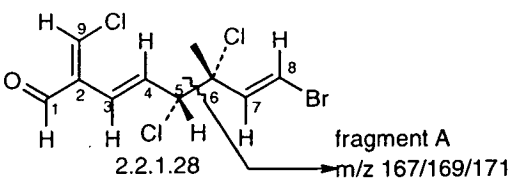
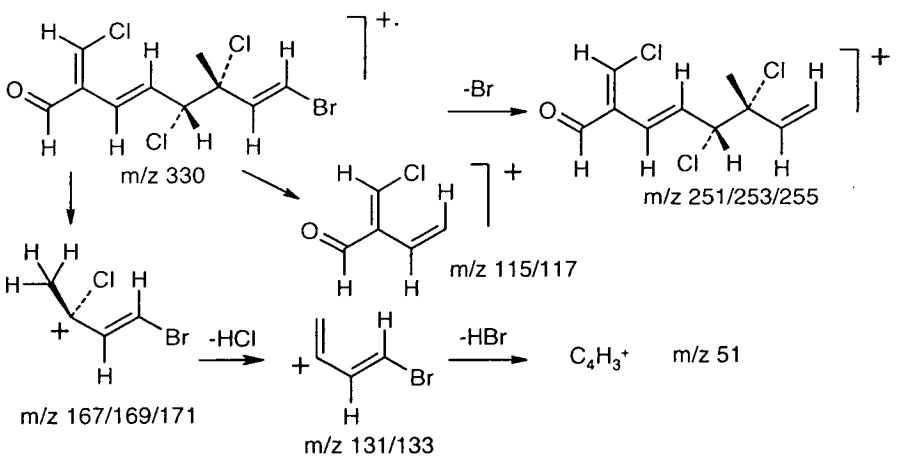


Table 2.2.1.1 ^{1}H (200 MHz for (2.1.1.28) and 400 MHz for (2.1.1.29)) and ^{13}C (100 MHz for both compounds) NMR data in $CDCl_3$

No	(2.1.1.28)		(2.1.1.29)	
	^{1}H, *J* (Hz)	^{13}C, DEPT	^{1}H, *J* (Hz)	^{13}C, DEPT
1	9.55 d, 2.0	189.3, CH	6.75 d, 0.6	124.6, CH
2	-	137.4, C	-	136.0, C
3	6.55 ddd, 0.9, 2.1, 15.9	123.0, CH	6.60 d, 16.1	124.4, CH
4	7.01 ddd, 0.6, 8.9, 15.9	133.7, CH	6.42 ddd, 0.7, 8.6, 16.2	137.0, CH
5	4.54 dd, 0.8, 8.8	69.5, CH	4.55 dd, 0.7, 8.6	69.2, CH
6	-	71.6, C	-	71.9, C
7	6.46 d, 13.5	136.9, CH	6.45 d, 13.3	131.5, CH
8	6.61 d, 13.6	110.7, CH	6.58 d, 13.5	110.9, CH
9	7.15 s	144.3, CH	6.37 s	69.6, CH
10	1.82 s	28.0, CH_3	1.80 s	28.1, CH_3



Scheme 2.2.1.1 Proposed MS fragmentation of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

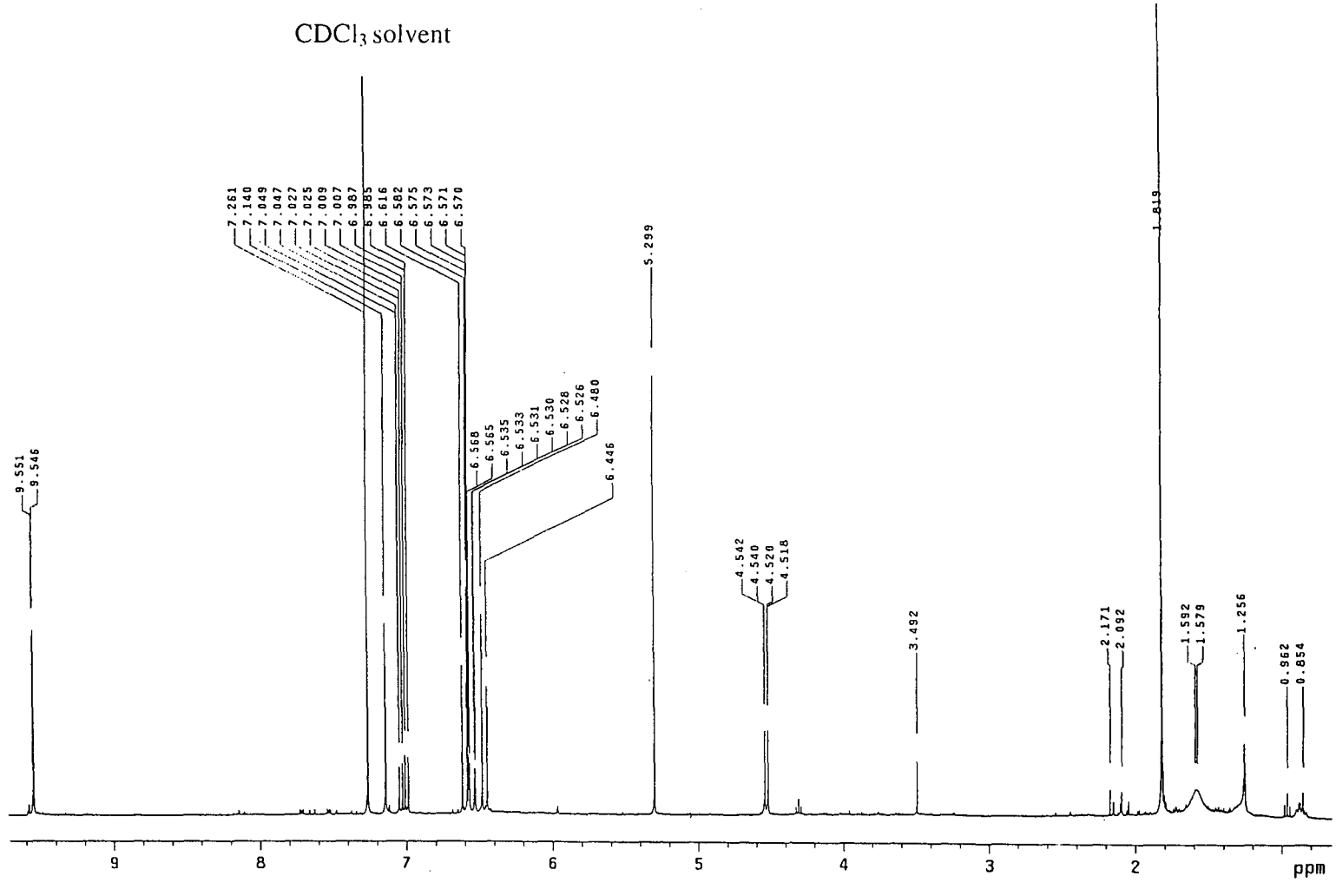


Figure 2.2.1.1 ^{1}H NMR spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

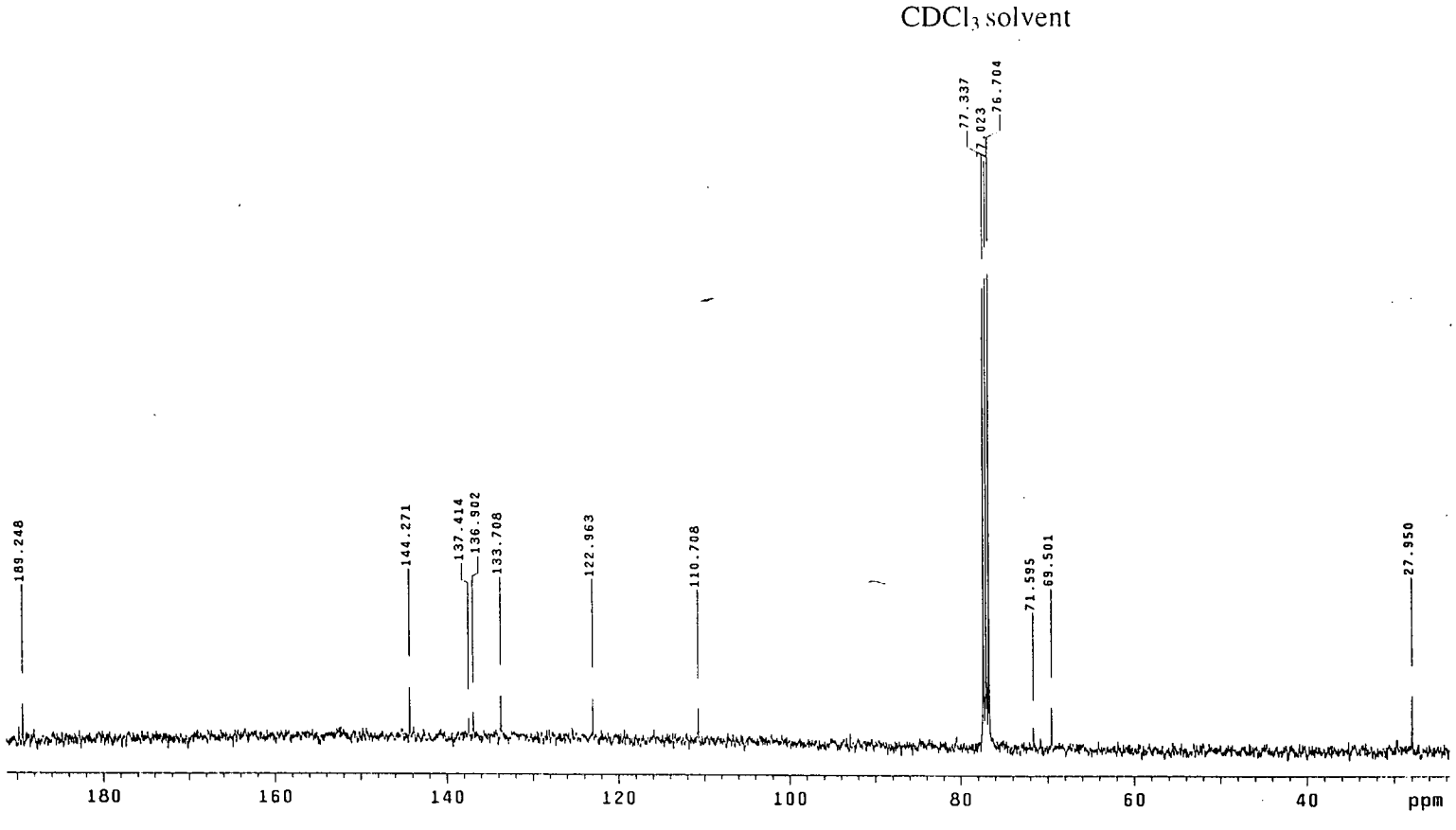


Figure 2.2.1.2 ^{13}C NMR spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

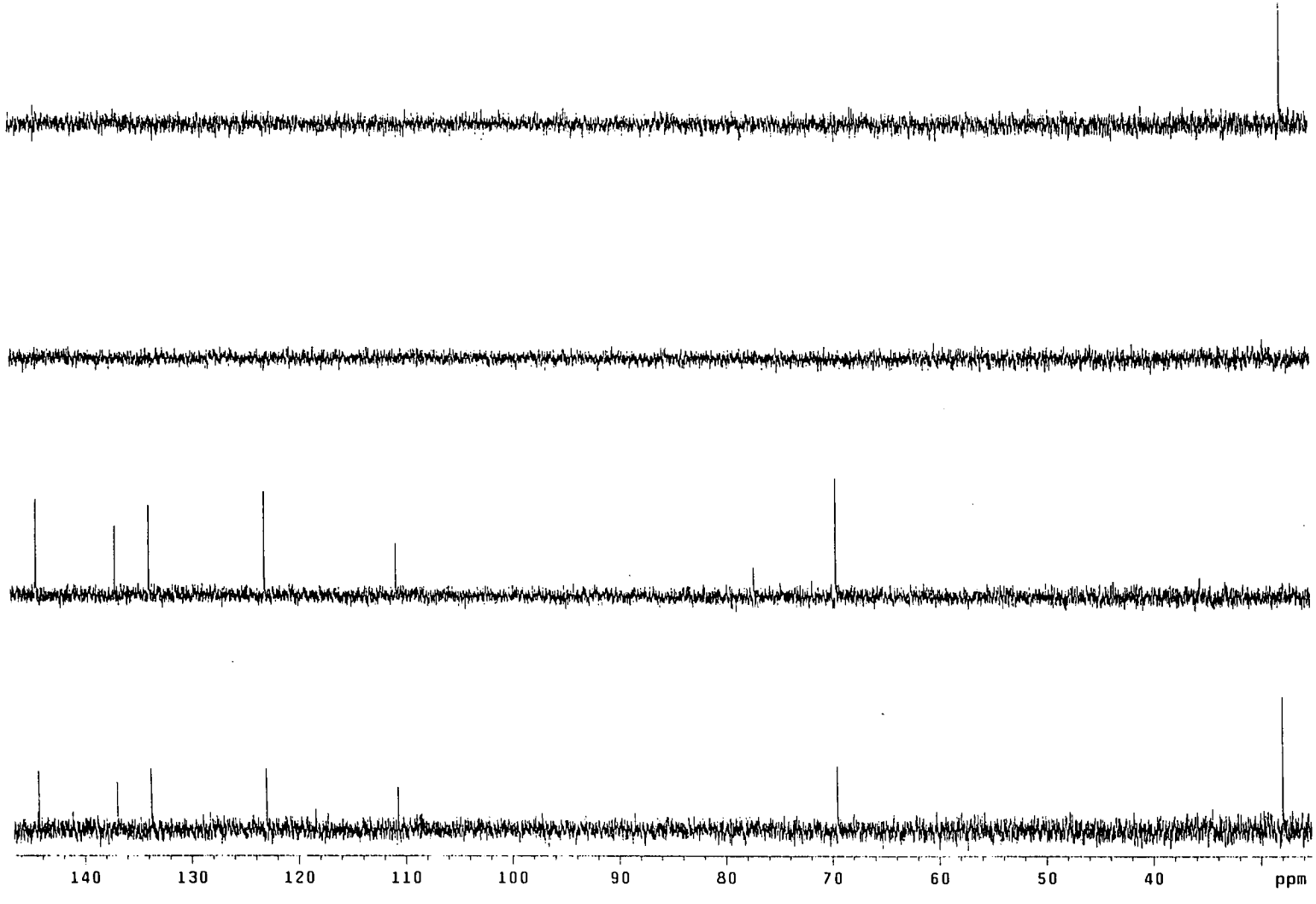


Figure 2.2.1.3 DEPT spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

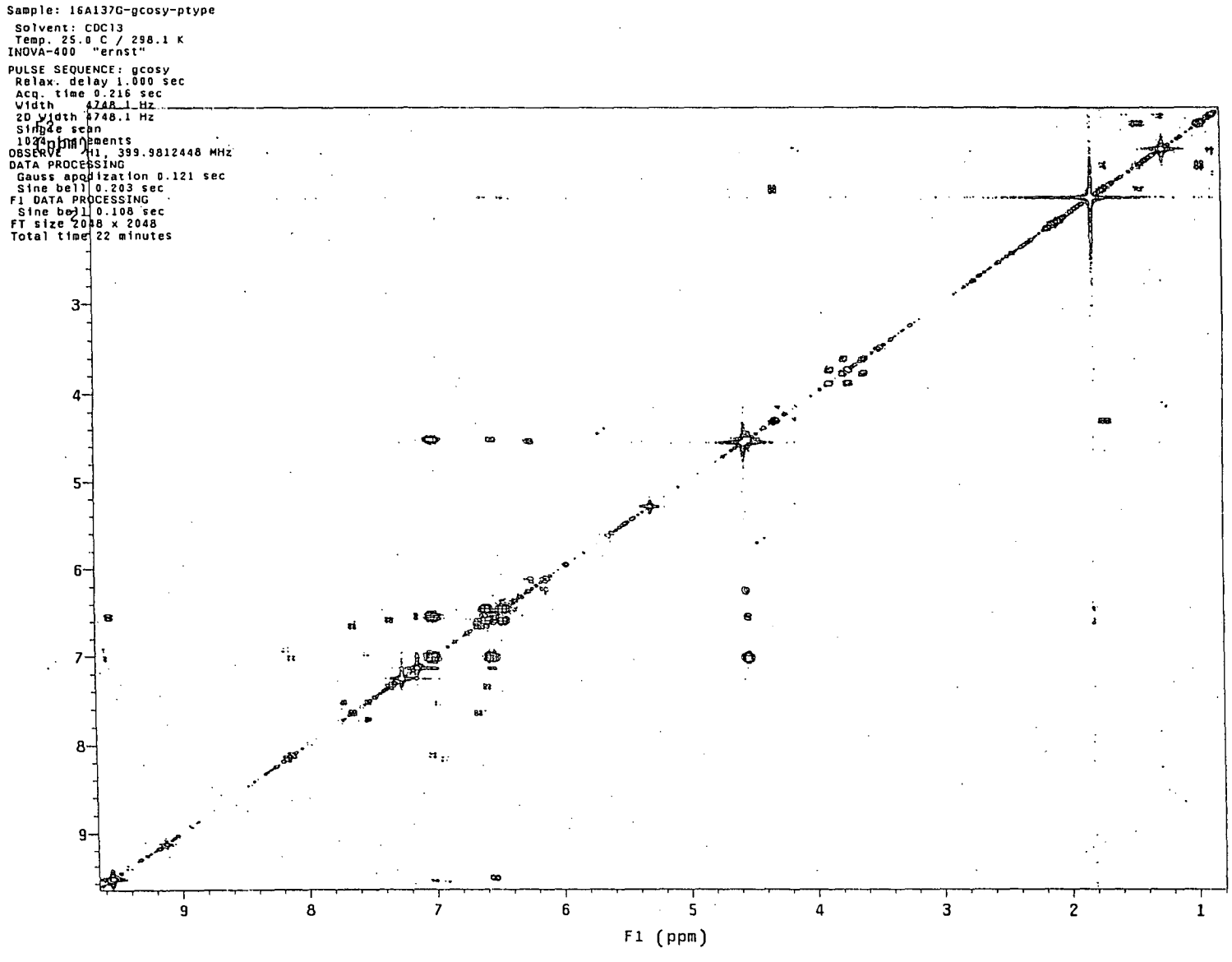


Figure 2.2.1.4 COSY spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

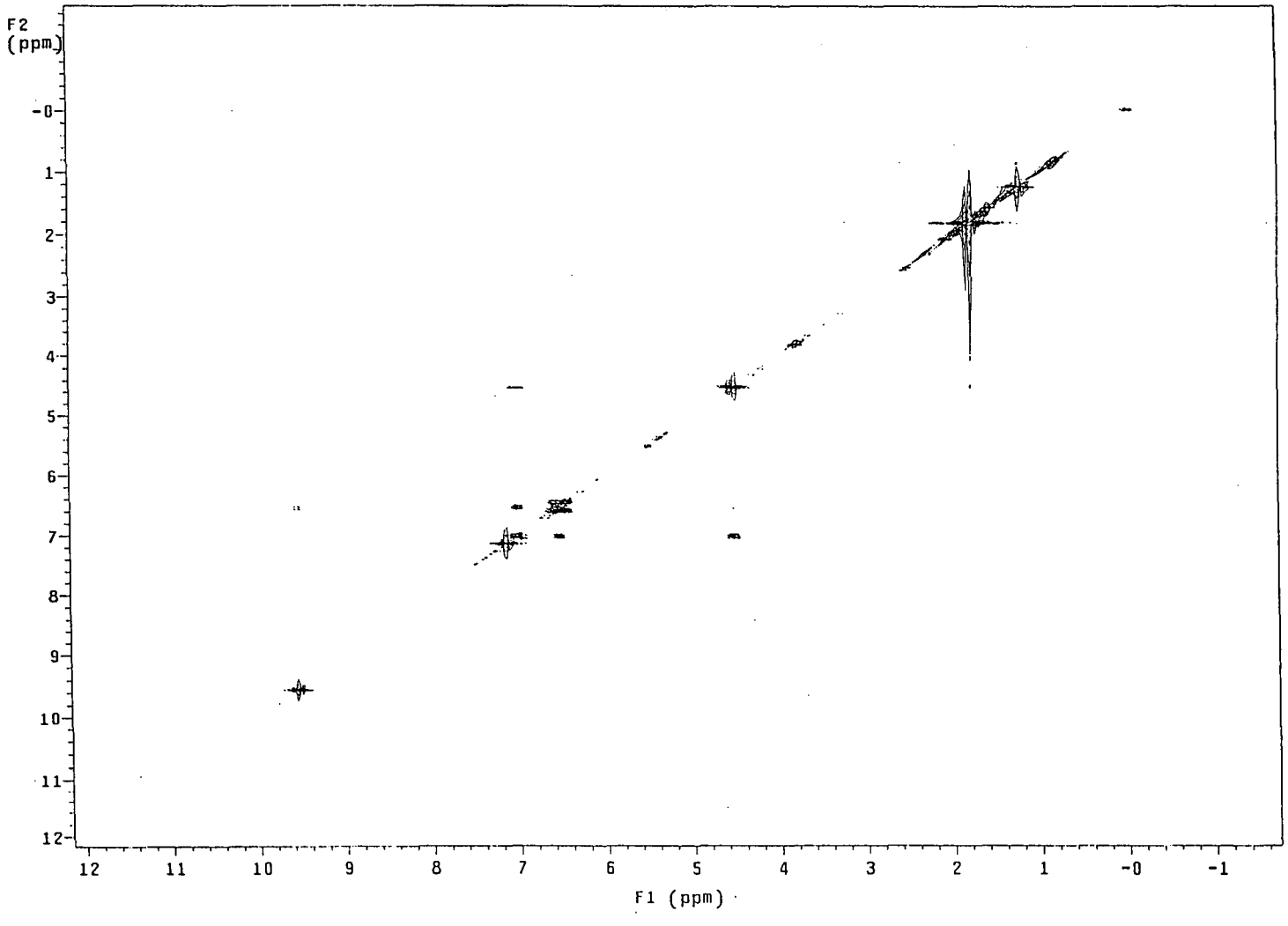


Figure 2.2.1.5 NOESY spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

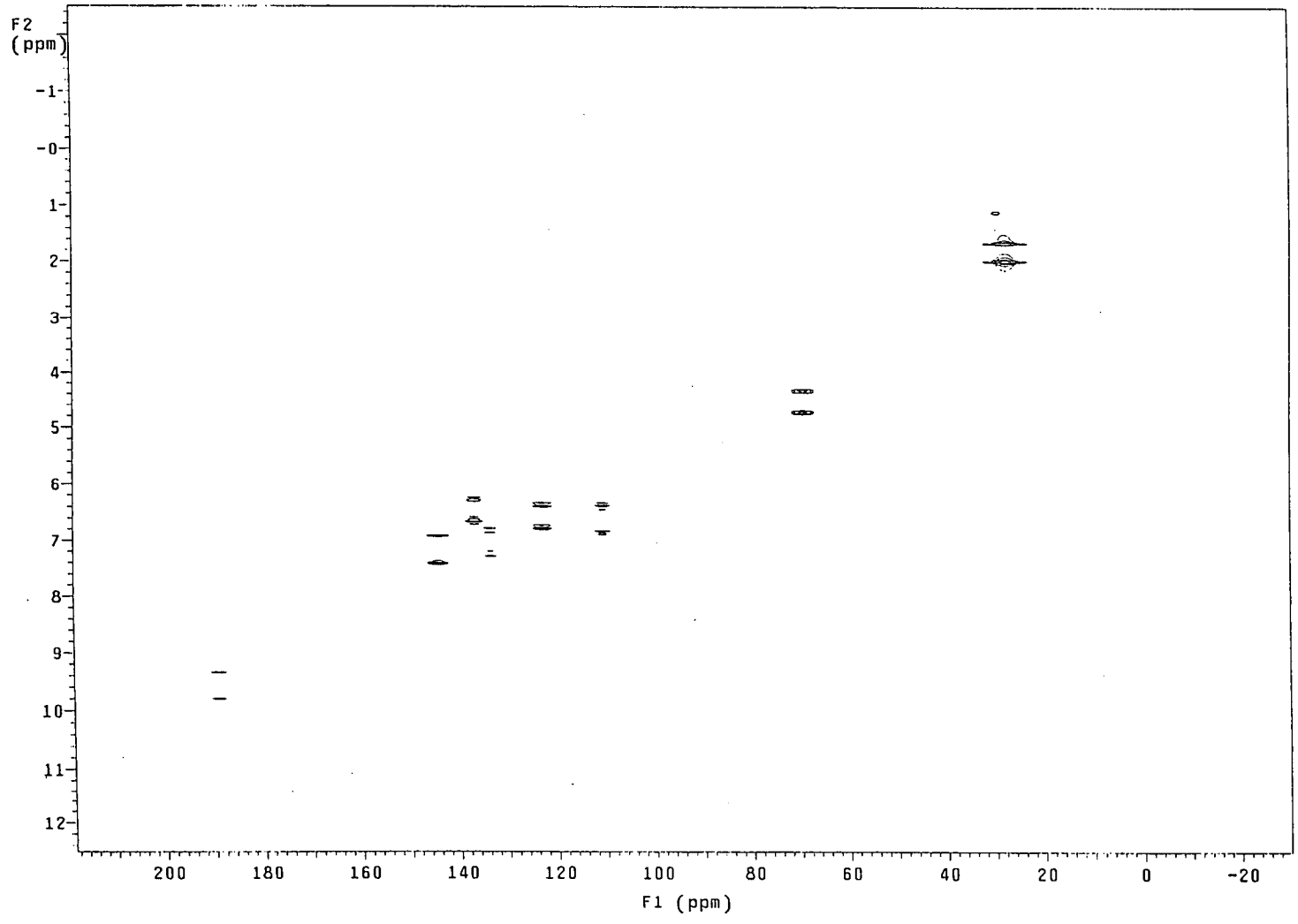


Figure 2.2.1.6 HMQC spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

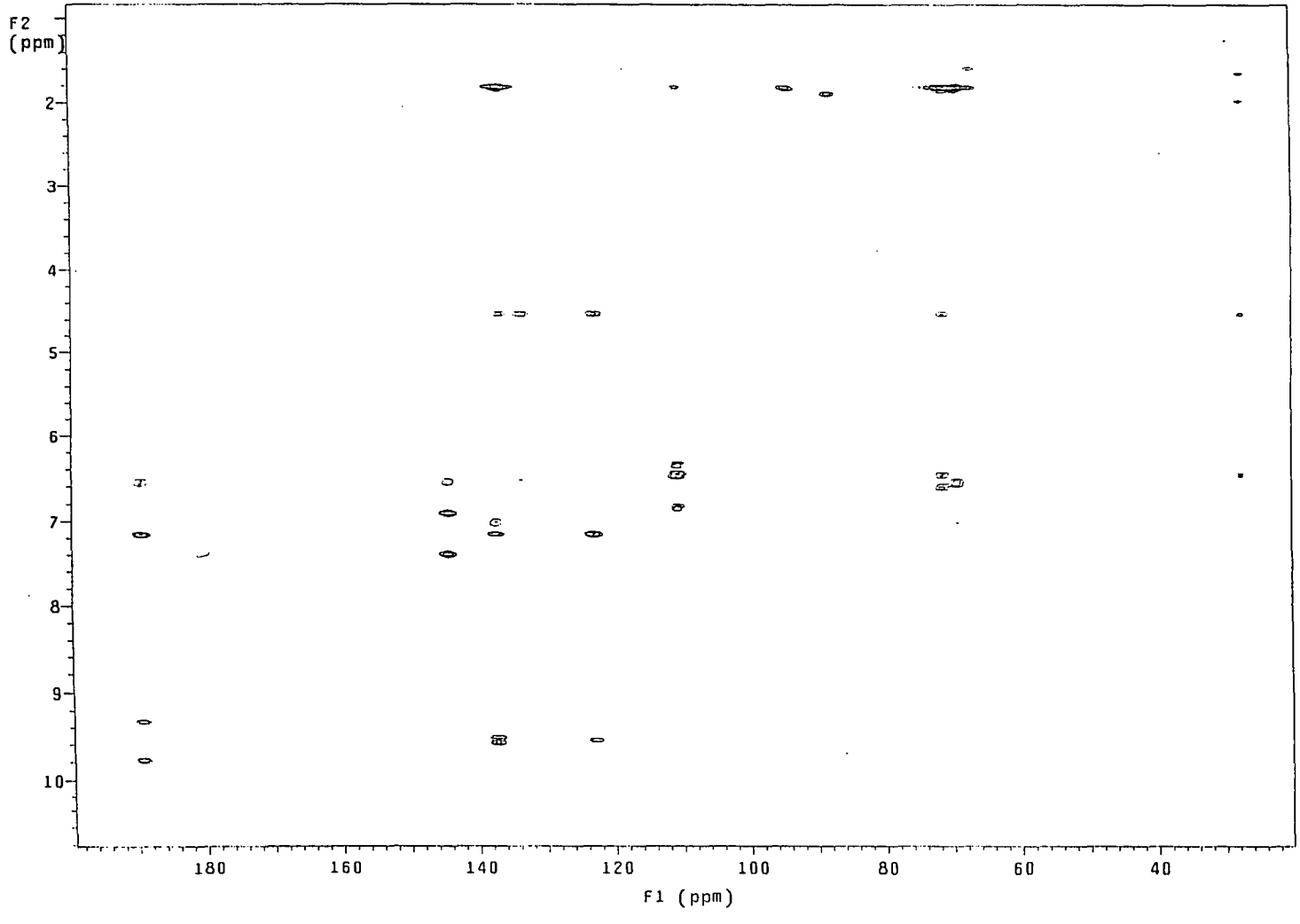


Figure 2.2.1.7 HMBC spectrum of (3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R**, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28).

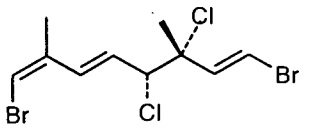
The geometry of the double bonds was determined by ^{1}H NMR spectroscopy. Two of the double bonds were 1,2-disubstituted and the coupling constants of the corresponding hydrogens revealed that they had a *trans* arrangement as mentioned earlier. The remaining double bond between C-2 and C-9 was attributed to the *E* isomer[34] by comparison of the observed chemical shift for H-9 at 7.15 ppm with the calculated chemical shift values from a table of substituent constants[35] which gave values for the *Z* isomer of 7.42 ppm and the *E* isomer of 7.01 ppm. The absence of an NOE between H-9 and any proton nearby and the absence of any coupling by H-9 in the ^{1}H NMR spectrum also supported the stereochemical assignment.

Chiral centres at C-5 and C-6 of (2.1.1.28) were assigned the relative stereochemistry (5*R**, 6*R**) by applying the empirical rules of Mynderse and Faulkner[36] and Crews[34] to the proton and carbon chemical shifts of the methyl group (H-10, 1.82 ppm and C-10, 28.0 ppm).

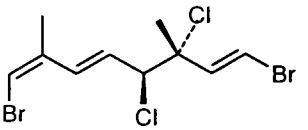
The empirical rule was first observed by Mynderse and Faulkner using ^{1}H NMR chemical shifts of methyl group (H-10) at the chiral C-6 in order to propose the relative stereochemistry of the acyclic polyhalogenated compound. The proton shift of 1.79 ppm was characteristic of 5(*R**) 6(*R**) whereas 1.73 ppm was characteristic of 5(*R**) 6(*S**). However, some compounds with shifts of 1.76 ppm could not be assigned to one of the two categories. Later Crews observed a much larger ^{13}C NMR methyl shift (C-10) difference of 28 ppm for 5(*R**) 6(*R**) and 25 ppm for 5(*R**) 6(*S**). The ^{13}C NMR methyl shift (C-10) difference of 3 ppm between the (*R*, *S*) and (*R*, *R*) configurations was observed and is much larger than the ^{1}H NMR methyl shift (H-10) difference of 0.06-0.08 ppm.[17, 33, 34, 36]

Compound (2.1.1.28), which had a positive optical rotation, $[\alpha]_D$+50.8^0 (*c* 0.128, CH_2Cl_2), was thus shown to be a 5,6-*threo* compound by using model compounds from an investigation of Mynderse and Faulkner.[36] The relative stereochemistry at C-5 and C-6 was assigned on the basis of observations from optical rotations and methyl group chemical shifts. Since compounds (2.2.1.1) and (2.2.1.2) were diastereoisomeric about C-5 and C-6 from the investigation by Mynderse and Faulkner. Compound (2.2.1.1) was assigned as 5,6-*threo* compound. In addition, compounds (2.2.1.1) and (2.2.1.3) both had a methyl signal at 1.79 ppm in the NMR spectrum and a positive optical rotation. While compounds (2.2.1.2) and (2.2.1.4) had the methyl signal at 1.73 ppm and a negative optical rotation. Compounds (2.2.1.3) and (2.2.1.4) were diastereomeric about C-5 and C-6. Compound

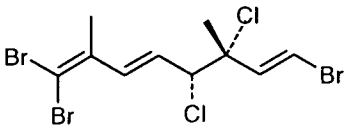
(2.2.1.3) was 5,6-*threo* whereas compound (2.2.1.4) was 5,6-*erythro*. Therefore, compound (2.1.1.28) was assigned to be 5,6-*threo* by using the same criteria of optical rotation and methyl group chemical shift. Unfortunately we do not have the authentic model samples (2.2.1.1-2.2.1.4) to compare directly with our compound (2.1.1.28).



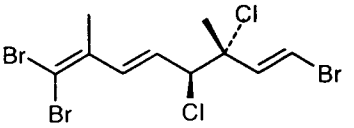
2.2.1.1



2.2.1.2

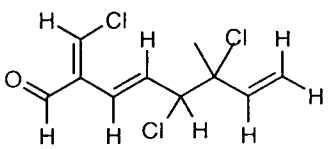


2.2.1.3



2.2.1.4

Compound (2.1.1.28) is the third acyclic halogenated monoterpene aldehyde that has been isolated from the *Plocamium* genus. All three are interestingly restricted to *P. cartilagineum*. The other aldehydes are cartilagineal (2.2.1.5)[34] and (3*Z*, 7*E*)-5, 8-dibromo-2, 6-dichloro-2, 6-dimethylocta-3, 7-dien-1-al (2.1.1.23).[17]



2.2.1.5

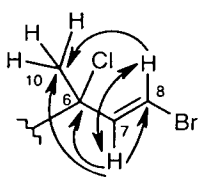
2.2.2 Structure of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

The EIMS showed molecular ion at m/z 428 with relative intensities for two bromine and four chlorine atoms (428 (0.013), 430 (0.105), 432 (0.137), 434 (0.068), 436 (0.028)), which correspond to the molecular formula of $C_{10}H_{10}Br_2Cl_4$ (HREIMS), indicating three degrees of unsaturation. The ^{13}C NMR spectrum of the compound (2.1.1.29) (Figure 2.2.2.1, Table 2.2.1.1) showed signals for ten carbons. The DEPT spectra allowed assignment of one methyl, seven methines (four olefinic, two with attached chlorine, and one with an attached bromine and chlorine), and thus implying the presence of two quaternary carbons in this molecule.

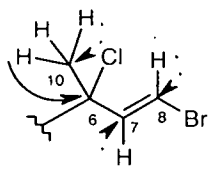
The base peak in the mass spectrum of (2.1.1.29), like that of compound (2.1.1.28), showed m/z 167/169/171. A proposed MS fragmentation is given in Scheme 2.2.2.1).

The ^{1}H NMR spectrum (Figure 2.2.2.2 and data in Table 2.2.1.1, $CDCl_3$ as solvent) showed signals at 6.75, 6.60, 6.58, 6.45, and 6.42 ppm) corresponding to the ^{13}C NMR spectrum that displayed signals at 137.0, 136.0, 131.5, 124.6, 124.4 and 110.9 ppm indicating three double bonds.

HMBC established a connection of a saturated quaternary carbon (C-6) at 71.9 ppm to a methine olefinic proton (H-7) at 6.46 ppm. The downfield chemical shift at C-6 implied an attached chlorine atom. The methine proton (H-7) was linked to a methine olefinic carbon (C-8) at 110.9 ppm by HMBC. The chemical shift at 110.9 ppm was due to a vinylic carbon atom (C-8) bearing a bromine atom.[6, 33, 34] COSY correlation between H-7 and H-8 was detected. The coupling constant ($J_{7,8}$ 13.2 Hz) between H-7 and H-8 supported a *trans* geometry. The carbon (C-6) was also connected to a methine olefinic proton (H-8) at 6.60 ppm by HMBC. Likewise, the proton (H-7) was linked to the carbon (C-10) by HMBC.

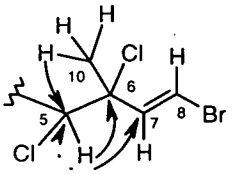


The carbon (C-6) was also linked to methyl protons $(H\text{-}10)_3$ at 1.82 ppm by HMBC. HMQC confirmed connections of C-7 to H-7, C-8 to H-8 and C-10 to $(H\text{-}10)_3$. Thus, the fragment A of this compound (2.1.1.29) was the same as in the compound (2.1.1.28).

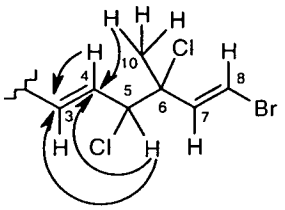


fragment A

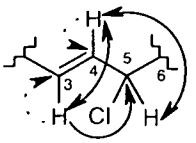
Connectivities by HMBC of the carbon (C-6) to a saturated methine proton (H-5) at 4.57 ppm and the methyl protons $(H\text{-}10)_3$ to a saturated methine carbon (C-5) at 69.2 ppm were shown. Like compound (2.1.1.28), the chemical shift of (2.1.1.29) at 69.2 ppm showed that C-5 had an attached chlorine atom.[6, 33, 34] HMQC confirmed a connection between C-5 and H-5. Moreover, the proton (H-5) was connected to the carbon (C-7) by HMBC.



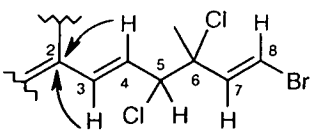
Next, the proton (H-5) was linked to a methine olefinic carbon (C-4) at 137.0 ppm and to another methine olefinic carbon (C-3) at 124.4 ppm by HMBC. Then the carbon (C-4) was connected to the methyl protons $(H\text{-}10)_3$ and the carbon (C-3) was connected to the proton (H-4) by HMBC.



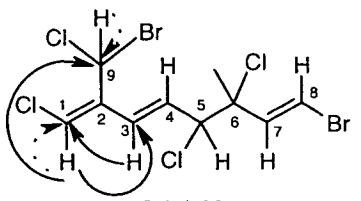
Similarly, the proton (H-3) was linked to the carbon (C-5) by HMBC and HMQC confirmed linkages between C-3 to H-3 and C-4 to H-4. COSY correlations revealed connections of H-3 to H-4 and H-4 to H-5. The coupling constant ($J_{3,4}$ 16.1 Hz) between H-3 and H-4 supported a *trans* geometry for the double bond.



Connectivities by HMBC of the proton (H-3) to an olefinic carbon (C-2) at 136.0 ppm and the carbon (C-2) to the proton (H-4) were established.

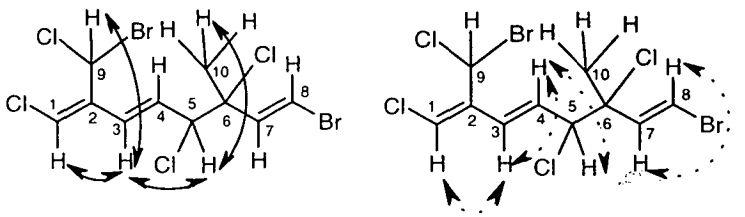


Likewise, HMBC connections of the proton (H-3) to a methine olefinic carbon (C-1) at 124.6 ppm, and the carbon (C-3) to a methine olefinic proton (H-1) at 6.76 ppm, as well as the proton (H-1) to a saturated methine carbon (C-9) at 69.6 ppm. HMQC confirmed connections between the carbon (C-9) to a saturated methine proton (H-9) at 6.76 ppm and C-1 to H-1. The chemical shifts of both proton and carbon at position 1 indicated an attached chlorine atom.[6, 17, 33] The saturated methine carbon (C-9) was thus attached to chlorine and bromine atoms; the chemical shift of C-9 at 69.6 ppm supported this halogen attachment.

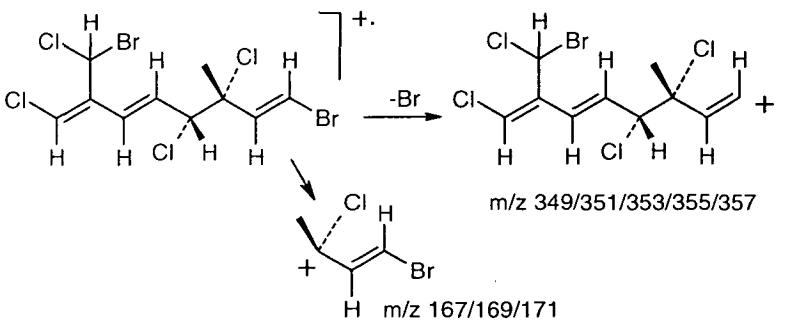


2.1.1.29

COSY correlations between H-1 to H-3, H-3 to H-5, H-3 to H-9, and H-5 to $(H\text{-}10)_3$ were demonstrated. NOESY correlations between H-1 to H-3, H-3 to H-4, H-4 to H-5, and H-7 to H-8 were revealed.



The stereochemistry of the two 1,2-disubstituted double bonds was assigned as *trans* on the basis of coupling constants ($J_{3,4}$ 16.1 and $J_{7,8}$ 13.3 Hz). The stereochemistry of the remaining double bond (C-1 and C-2), was allocated a *Z* geometry on the basis of an NOE between H-1 and H-3.



Scheme 2.2.2.1 Proposed MS fragmentation of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

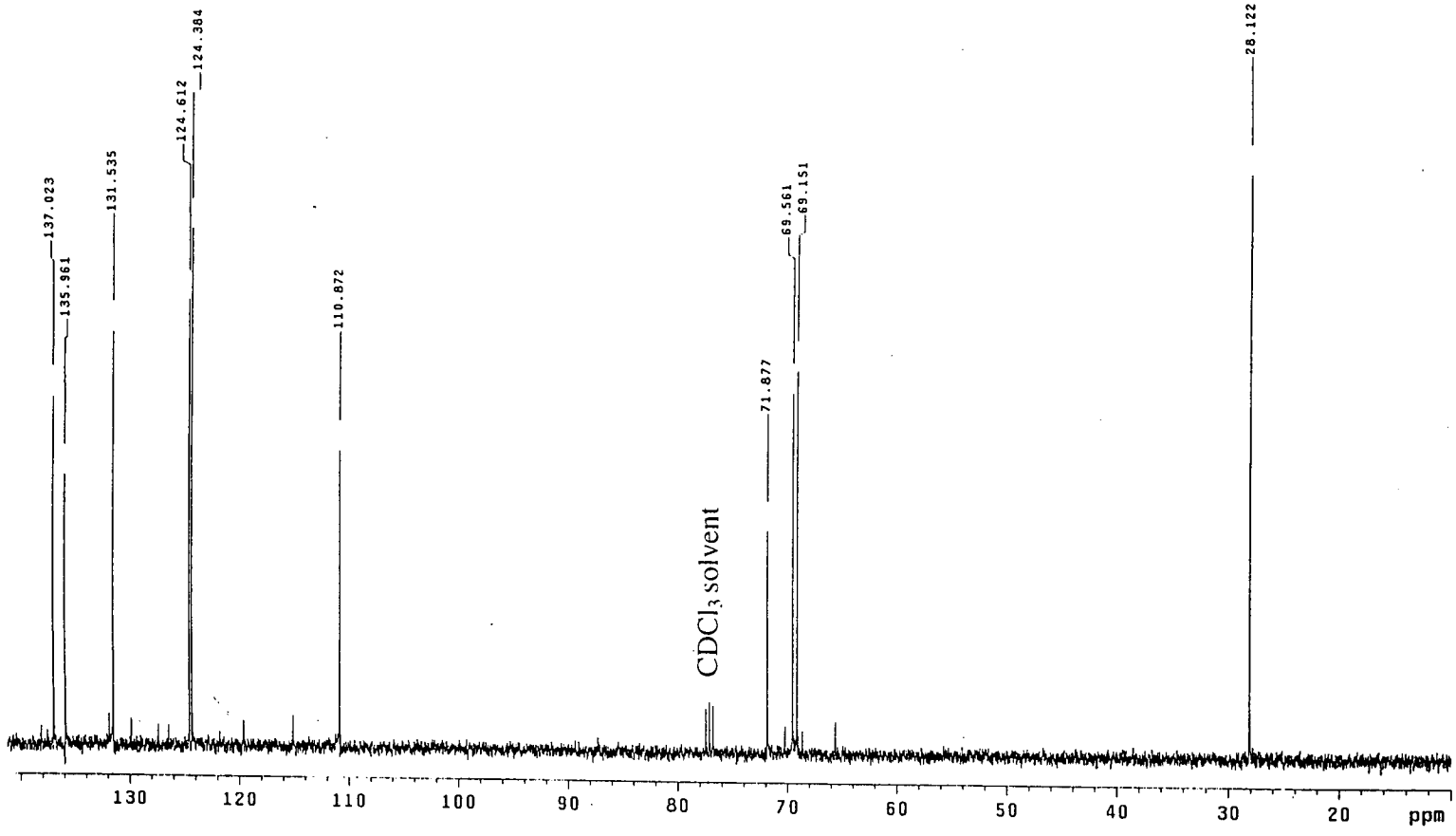


Figure 2.2.2.1 ^{13}C NMR spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

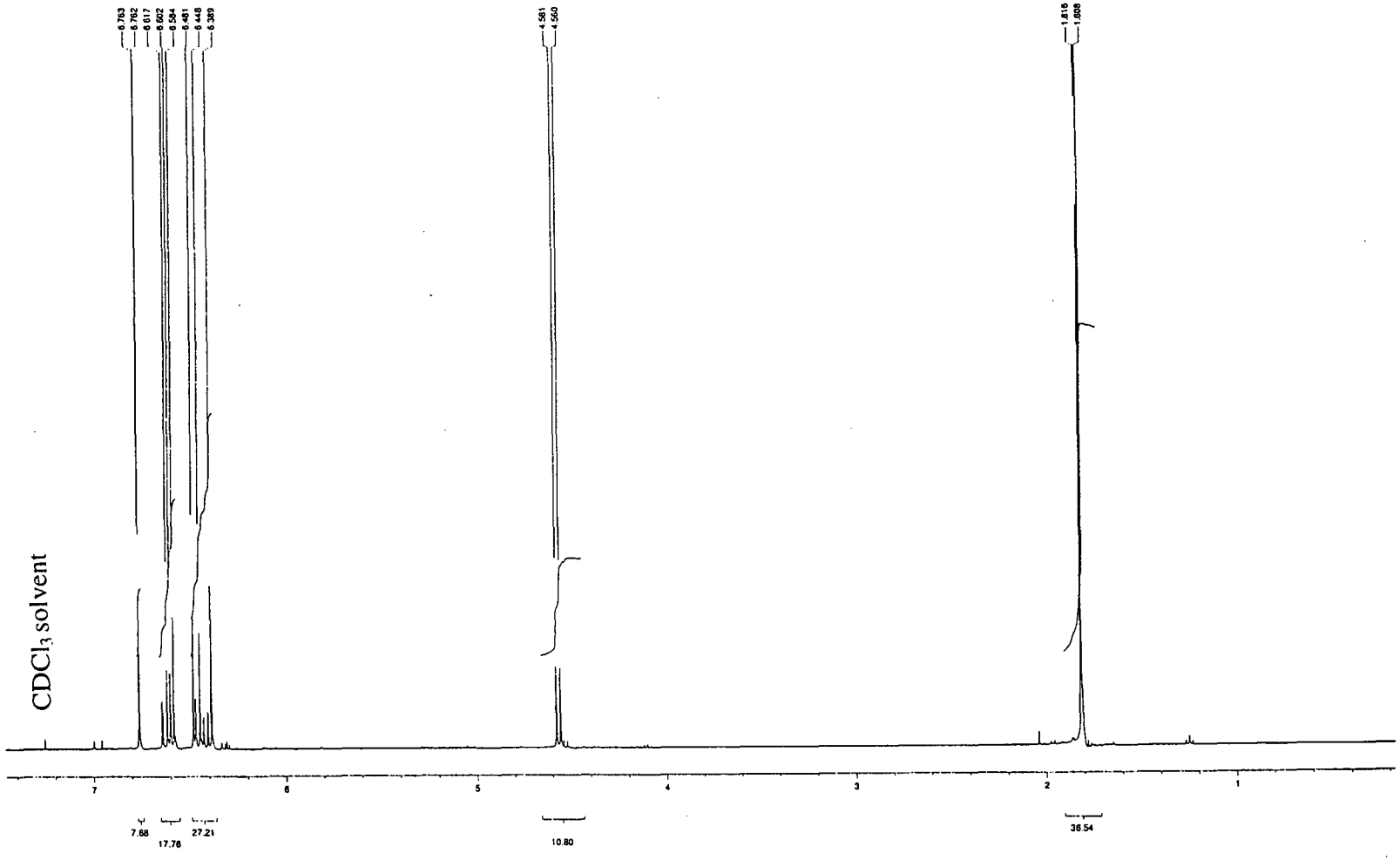


Figure 2.2.2.2 ^{1}H NMR spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

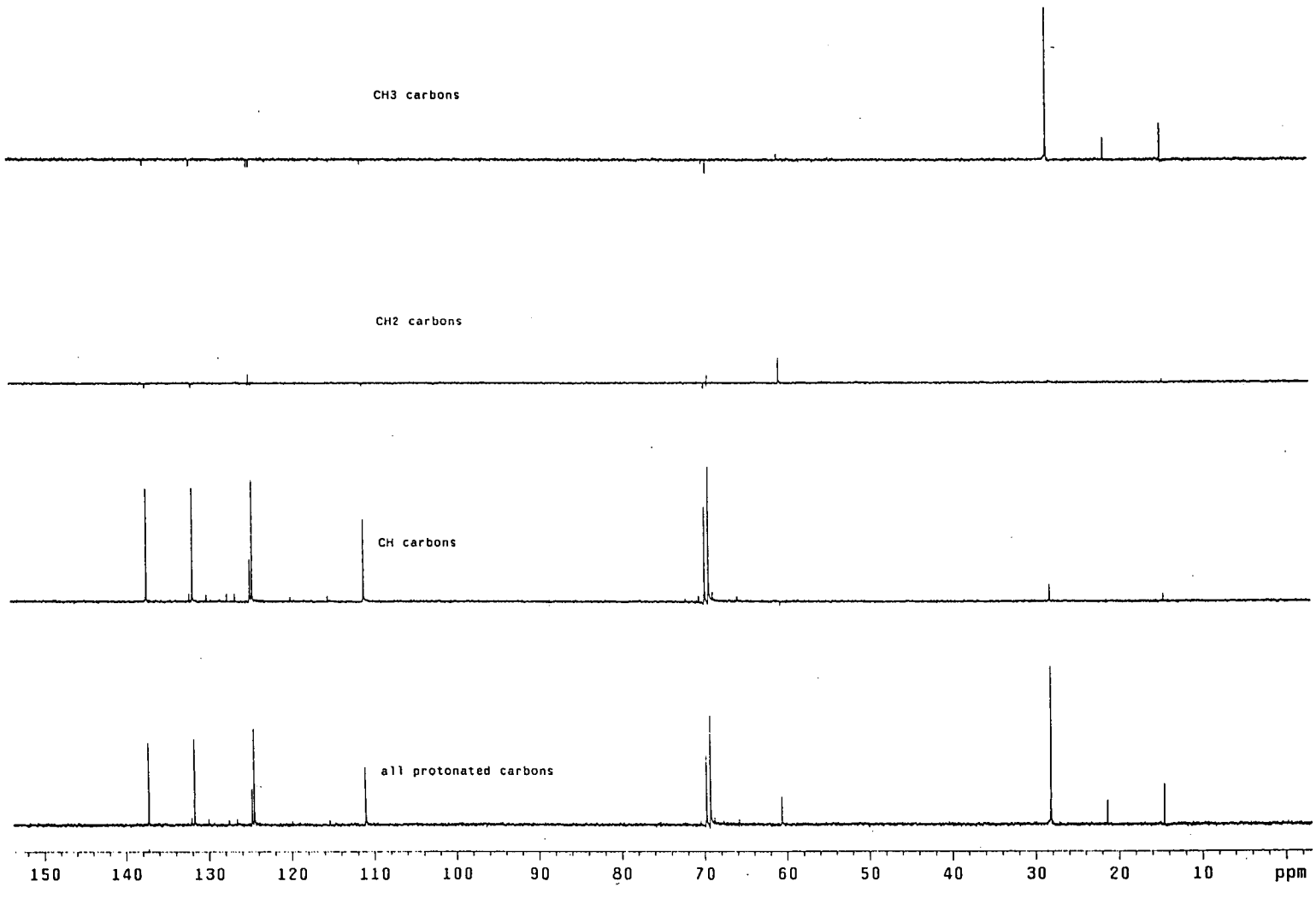


Figure 2.2.2.3 DEPT experiment of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

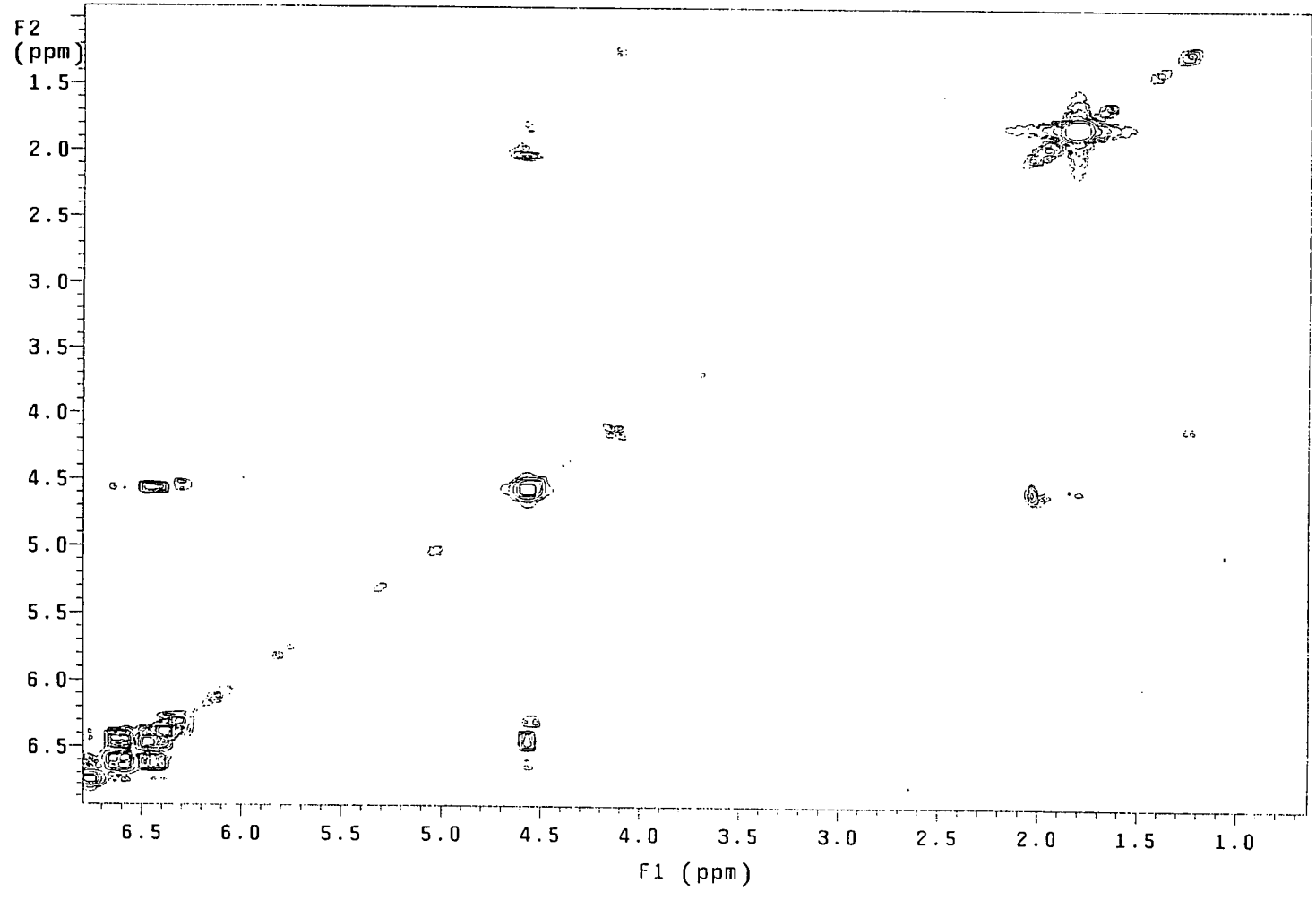


Figure 2.2.2.4 COSY spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

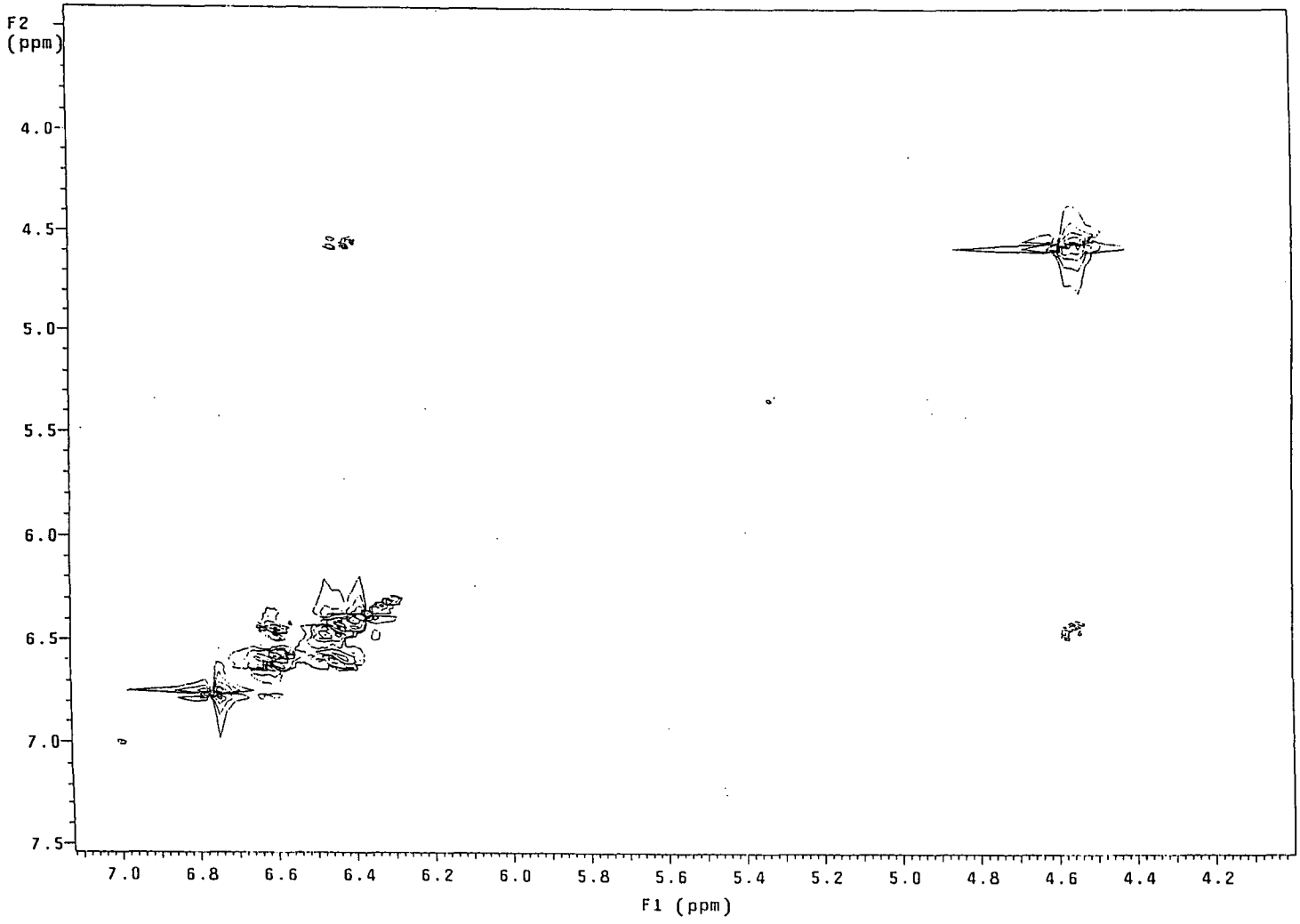


Figure 2.2.2.5 NOESY spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

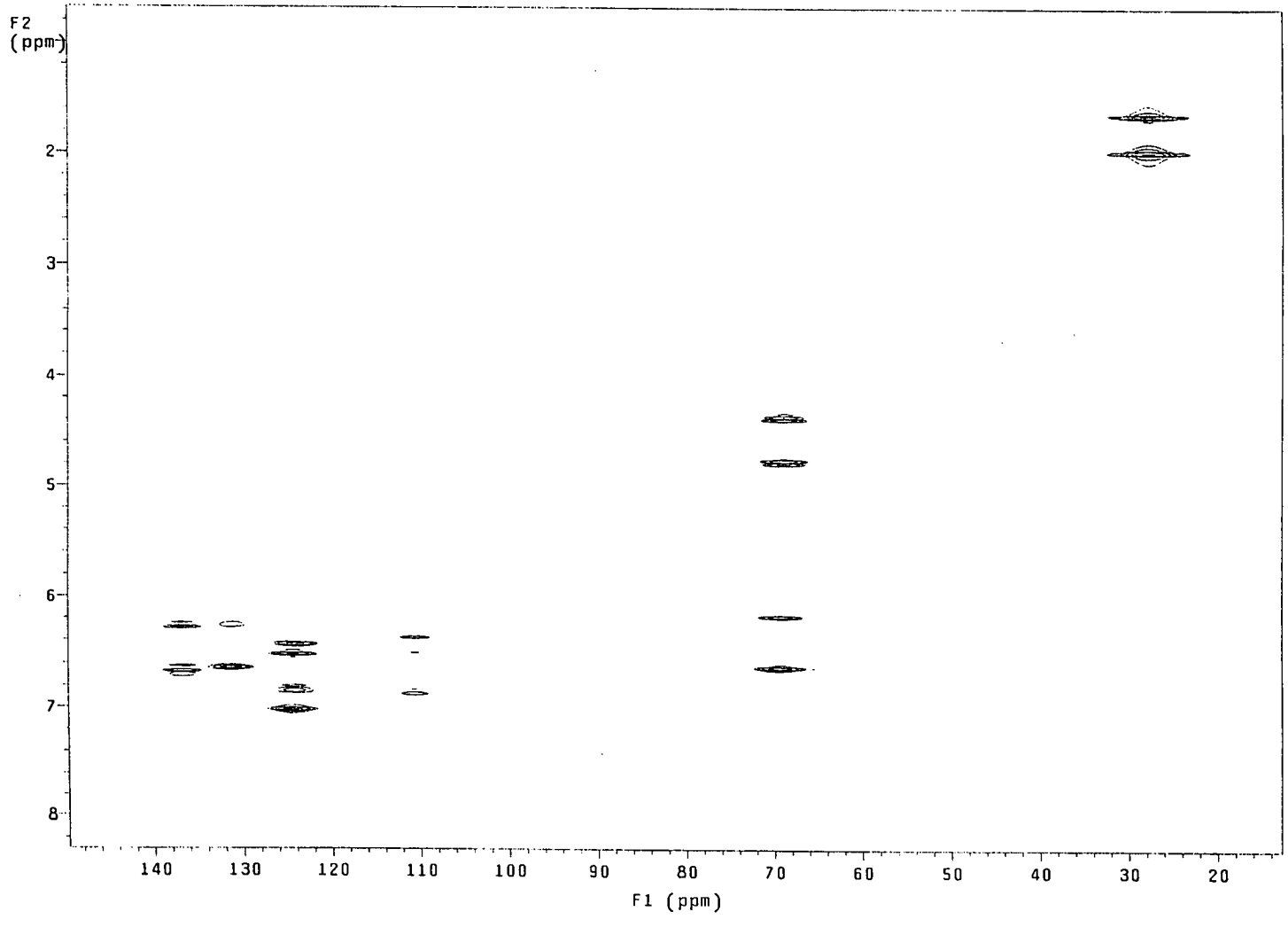


Figure 2.2.2.6 HMQC spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

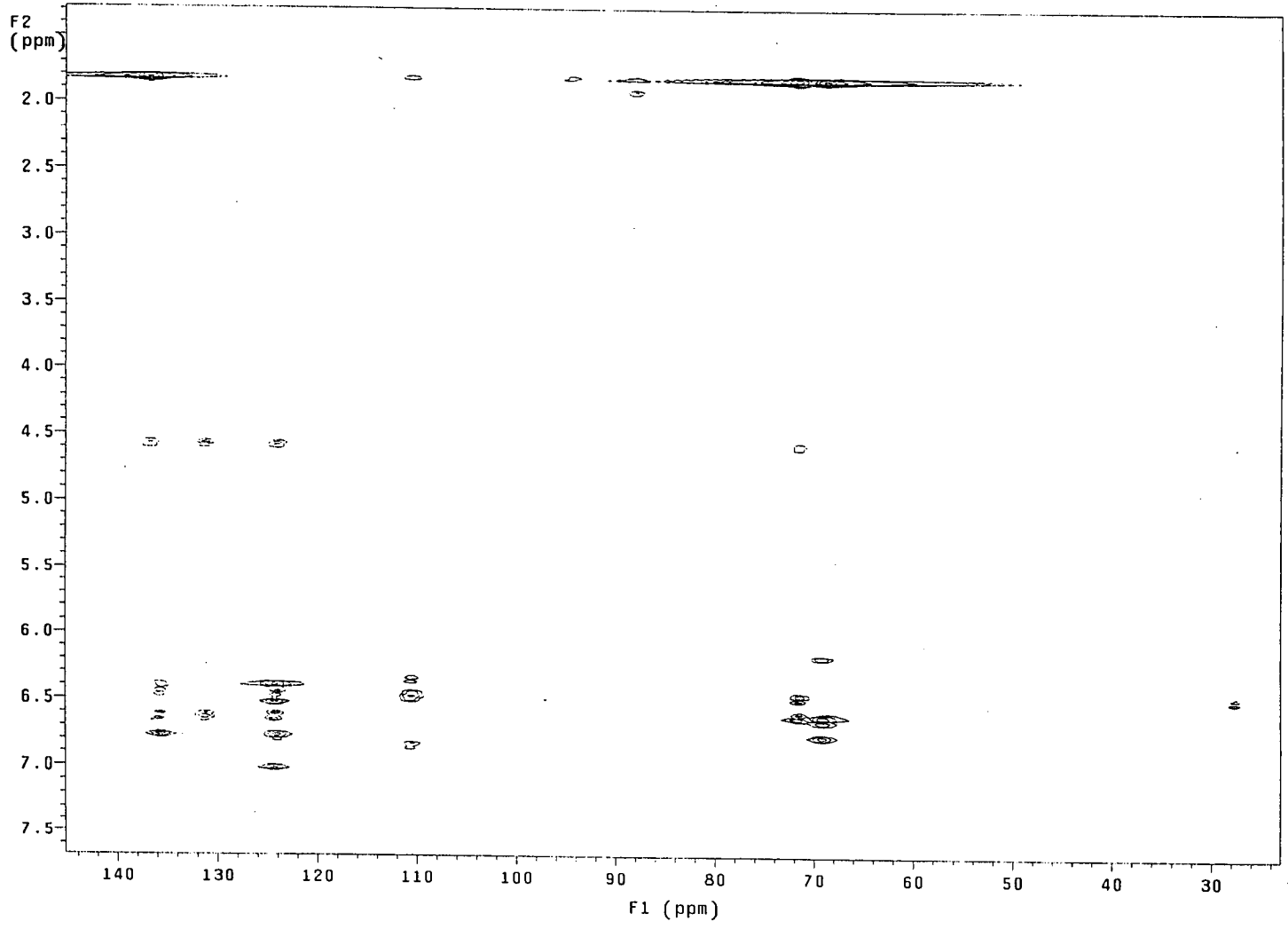
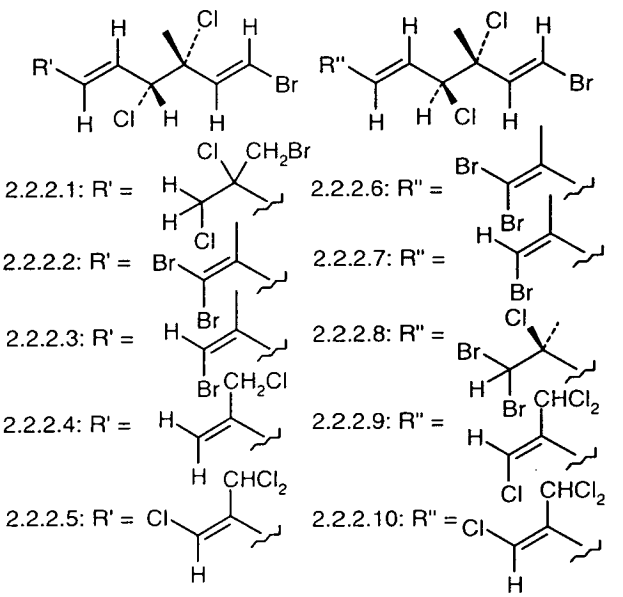


Figure 2.2.2.7 HMBC spectrum of (1*Z*, 3*E*, 7*E*)-8,9-dibromo-(1*Z*, 5*R**, 6*R**, 9)-tetrachloro-6-methyloctatriene (2.1.1.29).

The chiral centres at C-5 and C-6 of (2.1.1.29) were assigned the relative stereochemistry (5*R**, 6*R**) by using the methyl chemical shift rule (H-10, 1.80 and C-10, 28.0 ppm).[34, 36] Compound (2.1.1.29), which had a positive optical rotation, [α_D +49.1^0 (*c* 1.120, CH_2Cl_2), was thus shown to be a 5, 6-*threo* compound,[36] the same as compound (2.1.1.28).

These two new compounds (2.1.1.28-2.1.1.29) reported in this study have the same subunit from C-3 to C-8 as the two known compounds in this study (2.1.1.30-2.1.1.31) (by GCMS spectrum, Figure 2.2.2.7 and 2.2.2.8), and the other known compounds before 1984 (2.2.2.1-2.2.2.10).[25, 27, 33] Five of them had the same stereochemistry as (2.1.1.28-2.1.1.29).



2.3 Conclusion

Comparison of the two collections revealed that both contained the same metabolites, the two new (2.1.1.28-2.1.1.29) and two known (2.1.1.30-2.1.1.31) compounds. No significant decomposition occurring in the second collection, obtained from a beach shortly after a storm. The only detectable difference in the latter collection was that it also contained elemental sulfur (isolated as crystals). The sulfur was produced while it lay in heaps on the beach by anaerobic bacteria. Sulfur is a known metabolite of chemosynthetic bacteria. For example sulfur is produced by *Thiobacillus thioparus*. If aerobic conditions occur, or by photosynthetic bacteria under continued anaerobiosis, can oxidised substrates such as thiosulfate or tetrathionate to sulfate while at the same time depositing the reduced form of elemental sulfur.[37] Although *Plocamium cartilagineum* has a world-wide distribution and is restricted to temperate seas, it is of interest that this Tasmanian collection afforded new metabolites (not seen elsewhere) and provides another example of geographical variability in secondary metabolites from individual species.

The brine shrimp bioassay result showed one hundred percent mortality of the third dry-column flash chromatography fraction containing mainly the acyclic halogenated monoterpenes of *Plocamium cartilagineum* at concentrations of 92.5 μg/mL with *Artemia salina*. This study showed considerably higher activity than the previous report as 500 μg/mL for acyclic halogenated terpenes from *Plocamium* species.[16, 18]

2.4 Experimental.

2.4.1 Collection.

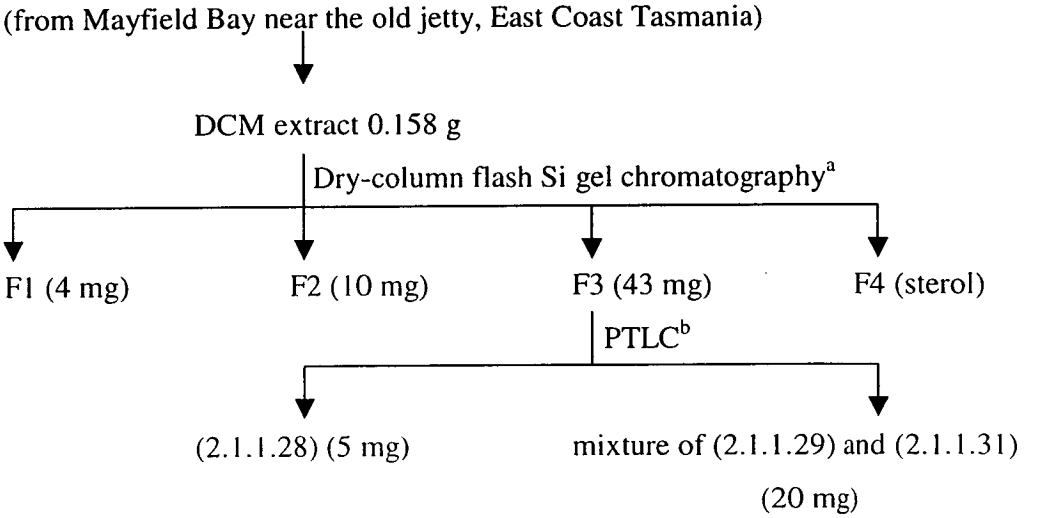
The red seaweed *Plocamium cartilagineum* was firstly collected by scuba diving at a depth of 5 m from Mayfield Bay, near the old jetty (42° 15' S, 148° 0.9' E) in November, 1997 and secondly gathered on Schouten Beach, Swansea (42° 7.7' S, 148° 5' E) in April, 1998. Both reference specimens have been lodged at the Tasmanian State Herbarium, Hobart (HO 445478 and HO 444898, respectively).

2.4.2 Extraction procedure.

The red seaweed material was sorted out, frozen and freeze-dried. The dried samples (20.626 g and 2 kg from the first and second collection, respectively) were exhaustively extracted with dichloromethane. The dichloromethane extracts were

concentrated on a rotary evaporator at a temperature below 30 °C to give a dark brown viscous tar (0.158 g and 52.2 g, respectively).

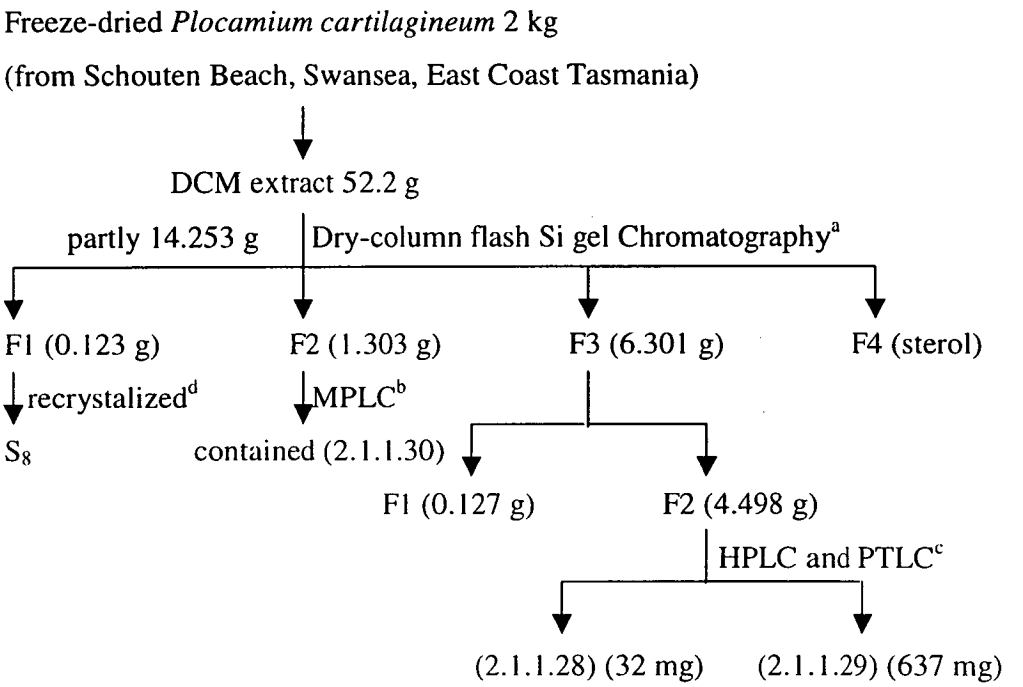
2.4.3 Separation procedure.



[a]using petroleum ether with increasing proportions of EtOAc gradually as eluent

[b]10% EtOAc/petroleum ether

Scheme 2.4.3.1 Separation procedure used for Mayfield Bay collection of *P.cartilagineum*.



[a]using petroleum ether with increasing proportions of EtOAc gradually as eluent

[b]10% EtOAc/petroleum ether

[c]0-10% EtOAc/petroleum ether for Si gel preparative HPLC and 10% EtOAc/petroleum ether for PTLC

[d]recrystallization in petroleum ether

Scheme 2.4.3.2 Separation procedure used for Schouten Beach collection of *P. cartilagineum*.

2.4.4 Characterization of acyclic halogenated monoterpene (2.1.1.28) and (2.1.1.29)

(3*E*, 7*E*)-8-bromo-2*E*-chloromethylene-5*R, 6*R**-dichloro-6-methyloctadiene-1-al (2.1.1.28)** was isolated as a pale yellow oil; $[\alpha]_D$+50.8^0 (*c* 0.128, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 261 (3.98), 234 (3.98) nm; IR (Nujol) ν_{max} 1726, 1055, 939 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2.2.1.1; CIMS m/z found 347.9310 $[M+NH_4]^+$ (calcd for $C_{10}H_{14}{}^{79}Br^{35}Cl_3NO$ obs. 347.9324); EIMS m/z 295 ($[M-Cl]^+$, 1.9), 297 (3.4), 299 (1.4), 301 (0.3), 251 ($[M-Br]^+$, 2.6), 253 (2.2), 255 (0.9), 215 ($[M-HBr-Cl]^+$, 4.1), 217 (2.7), 219 (4.6), 180 ($[M-HBr-2Cl]^+$, 7.7), 182 (7.5), 167 ($[C_4H_5BrCl]^+$, 79.9), 169 (100), 171 (26.3), 131 ($[C_4H_4Br]^+$, 18.7), 133 (16.7), 117 (21.2), 115 (34.3), 51 (36.9).

(1*Z*, 3*E*, 7*E*)-9-bromo-1*Z*, *5R, *6R**, *9*-tetrachloro-6-methyloctatriene (2.1.1.29)** was isolated as a viscous pale yellow oil; $[\alpha]_D$+49.1^0 (*c* 1.120, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 249 (5.31) nm; ^{1}H and ^{13}C NMR data, see Table 2.2.1.1; HREIMS m/z found 431.77949 $[M]^+$ (calcd for $C_{10}H_{10}Br_2Cl_4$ obs. 431.78559, average of all isotopes, due to the lowest isotope peak being very weak); EIMS m/z 428/430/432/434/436 M^+ (expansion of scale to see the pattern), 384/386/388/390 (expansion), 349 (2.0), 351 (4.9), 353 (4.2), 355 (1.6), 305 (1.4), 307 (2.2), 309 (1.5), 269 (1.0), 271 (1.5), 273 (8.4), 233 (2.1), 235 (3.7), 237 (2.2), 197 (1.9), 199 (5.3), 201 (3.1), 167 ($[C_4H_5BrCl]^+$, 80.0), 169 (100), 171 (26.1), 147 (8.7), 133 (16.5), 75 (18.4), 51 (44.3).

The GCMS spectra of the known compound (2.1.1.30) (m/z 361/363/365 expansion, 327 (0.3), 317 (1.4), 319 (1.7), 321 (1.5), 283 (0.7), 247 (1.5), 229 (1.7), 231 (1.9), 212 (1.4), 194 (6.2), 196 (5.4), 181 (2.9), 183 (2.9), 167 (82.0), 169 (100), 171 (24.9), 131 (14.8), 133 (20.3), 115 (29.2), 91 (16.3), 77 (24.9), 79 (24.2), 65 (13.1), 51 (23.3)) and compound (2.1.1.31) (m/z 293 (2.3), 295 (5.0), 297 (3.0), 270 (0.8), 249 (47.7), 251 (60.6), 253 (14.4), 237 (2.3), 213 (12.9), 215 (10.6), 205 (16.7), 207 (15.2), 195 (4.5), 167 (4.5), 169 (21.2), 171 (9.1), 161 (6.1), 133 (33.3), 119 (28.8), 103 (17.4), 105 (18.2), 91 (43.9), 77 (50.0), 79 (49.9), 53 (42.4), 41 (100)) were consistent with the literature.[33, 38, 39]

2.4.5 Bioassay.

Brine shrimp (*Artemia salina*) bioassay[40] was performed on the third dry-column flash chromatography fraction, which contained mainly the acyclic halogenated monoterpenes of *P. cartilagineum*. One hundred percent mortality was obtained at concentrations of 92.5 μg/mL or greater after 15 hours (see Table 2.4.5.1). This study showed considerably higher activity than the previous report (eg. 500 μg/mL) for acyclic halogenated terpenes from *Plocamium* species.[16, 18]

Table 2.4.5.1 Brine shrimp bioassay results for the third dry-column flash chromatography fraction of *P. cartilagineum*.

Concentration (μg/mL)	Percent deaths at 15 hrs[a]
control	10
12.3	60[b]
30.8	77[b]
61.7	93[b]
92.5	100
123.4	100
1,542	100
3,084	100
30,840	100

[a]average of 3 replicates.

[b]100% death after 20.5 hours.

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Chapter 3 *Aplysia parvula* and *Laurencia filiformis*.

Aplysia parvula is a herbivorous sea hare, which accumulates secondary metabolites from its diet. Various metabolites, both halogenated and non-halogenated compounds have been isolated from the sea hare *Aplysia*.[1,2] The sea hare *Aplysia* sp. secretes a purple ink when it is attacked, that can obscure it and distract predators.[3]

The red seaweed *Laurencia filiformis* was shown in this study to be the dietary source of most of the secondary metabolites found in the sea hare *Aplysia parvula*. Some examples of chemical ecology between the sea hare and red algae have been previously reported.[4,5,6,7] However, this study will attempt to describe the secondary metabolites from both organisms.

3.1 General introduction and secondary metabolites from *Aplysia*.

The herbivorous sea hare genus *Aplysia* is widespread in tropical to temperate waters around the world. *Aplysia parvula* Morch, 1863 that is the subject of this study belongs to phylum Mollusca, class Gastropoda, subclass Opisthobranchia, and family Aplysiidae. A characteristic of this species is the black trim to the parapodia and the black tips to the oral tentacles and rhinophores.[8]

Scheuer[9] reported sesquiterpenes, diterpenes, cyclic ethers, and a nitrogenous compound named aplysioviolin from the sea hare *Aplysia* sp. The author classified secondary metabolites from marine organisms into five major types, isoprenoids, sterols, benzenoids, nitrogenous compounds, and nonaromatic compounds with unbranched carbon skeletons. Barrow[10] also reported bile pigments and related compounds from algae and sea hares.

A comprehensive list of secondary metabolites from phylum Mollusca was presented by Alam *et al.*[11] This book covered the literature on molluscs published between 1976 and 1994. These compounds were arranged by molecular weight. The author classified all metabolites into nine types as follows: terpenes, polypropionates, aromatic nitrogenous compounds, aliphatic nitrogenous compounds, polypeptides, macrolides, prostaglandins and fatty acid derivatives, sterols, and miscellaneous compounds.

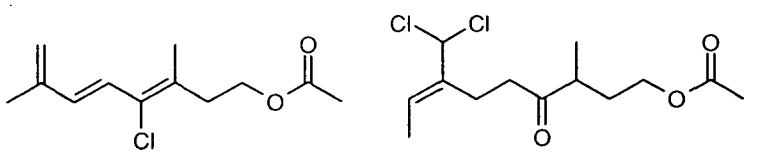
Yamada *et al.*[12] reported bioactive compounds from the sea hare *Aplysia* and *Dolabella* genera. The metabolites from the sea hare *Aplysia* were classified into main three groups, polyketides, terpenes, and others. The polyketides were aplyronines, aplydilactone,

and halogenated C_{15} cyclic ethers. The terpenes were monoterpenes, sesquiterpenes, diterpenes, and miscellaneous terpenes.

This review will discuss all secondary metabolites discovered in the sea hare genus *Aplysia* covering the literature published from 1995 to 2000 by SciFinder Scholar searching. In the case of the tetrapyrrole ink pigments from sea hares, this review will cover the literature published from 1966 to 2000.The focus will be on methods of isolation, structure determination and biological activity where possible. Secondary metabolites will be organised into structural types.

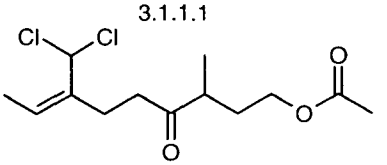
3.1.1 Polyhalogenated acyclic monoterpenes.

Ortega *et al.*[13] reported four new acyclic monoterpenes (3.1.1.1-3.1.1.4) from *Aplysia punctata*. Five specimens of the sea hare were collected by scuba diving in Sancti Petri, Cadiz, Spain. The specimens were cut into small pieces and extracted with acetone, followed by partitioning between diethyl ether and water. The organic soluble portion was purified by column chromatography SiO_2. The selected fractions were further purified with LiChrosorb HPLC to afford compounds (3.1.1.1-3.1.1.4, 3.1.2.1-3.1.2.4). Compounds (3.1.1.2-3.1.1.4) showed significant *in vitro* cytotoxicity with ED_{50} of 2.5 µg/mL against mice lymphoma (P388) and human colon carcinoma (HT29) cell lines and ED_{50} of 1.5 µg/mL against human lung carcinoma (A549) and human melanoma (MEL28) cell lines.

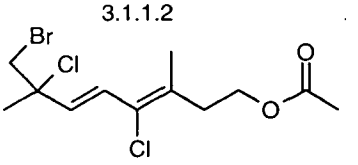


3.1.1.1

3.1.1.2



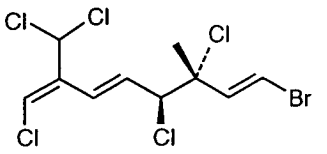
3.1.1.3



3.1.1.4

A known acyclic monoterpene (3.1.1.5) from *Aplysia dactylomela* was reported by Wessels *et al.*[14] One sample consisted of a single animal of *Aplysia dactylomela*, which was collected from the beach of San Juan de la Rambla, Tenerife, Canary Islands, Spain. Two samples of *Aplysia dactylomela*, one containing three animals and another consisting of a single animal, were collected at the same time from the ocean at Punta del Hidalgo, Tenerife, Canary Islands, Spain. The freeze-dried and extracted samples were purified on silica gel first using a vacuum liquid chromatography and then by HPLC to afford

metabolite (3.1.1.5). Both the single animal from the beach of San Juan de la Rambla and the single animal from the ocean at Punta del Hidalgo, Tenerife contained metabolite (3.1.1.5).

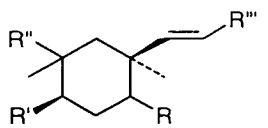


3.1.1.5

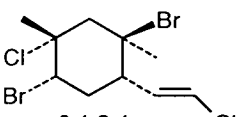
Compound (3.1.1.5) showed cytotoxicity against cultured cancer cell lines HMO2 gastric carcinoma, HEPG2 liver carcinoma, and MCF7 breast carcinoma at IC_{50} 1.1, 1.0 and 1.5 μg/mL, respectively. Moreover, compound (3.1.1.5) showed activity against bacteria *Bacillus megaterium* and showed an activity against the alga *Chlorella fusca*. However, this metabolite was not active against fungi *Eurotium repens*, *Fusarium oxysporum*, *Microbotryum violacea* and *Mycotypha microspora*, and brine shrimp *Artemia salina*.

3.1.2 Polyhalogenated cyclic monoterpenes.

In 1997 four known cyclic monoterpenes (3.1.2.1-3.1.2.4) from *Aplysia punctata* were also reported by Ortega *et al.*[13] The sea hare was collected from Sancti Petri, Cadiz, Spain.



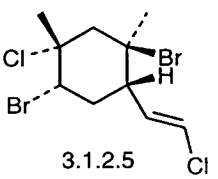
3.1.2.1 R= α-Br, R'= Br, R"= β-Cl, R"'= Cl
3.1.2.2 R= β-Br, R'= Cl, R"= α-Cl, R"'= Cl
3.1.2.3 R= β-Br, R'= Cl, R"= β-Cl, R"'= Br



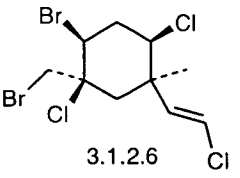
3.1.2.4

Wessels *et al.*[16] also reported two known cyclic monoterpenes (3.1.2.5-3.1.2.6) from *Aplysia dactylomela*. Three different samples of *Aplysia dactylomela* were collected from two different locations, namely San Juan de la Rambla and Punta del Hidalgo, Spain. Metabolite (3.1.2.5) was isolated from samples from both locations but compound (3.1.2.6) was only found from Punta del Hidalgo specimens. Compound (3.1.2.5) displayed activities toward the bacterium *Bacillus megaterium* and against fungi *Eurotium repens* and *Microbotryum violacea*, and toward brine shrimp *Artemia salina* at 90% lethality rate of a test concentration at 0.5 mg/mL after 48 hours. However, this metabolite was not active against the alga *Chlorella fusca*. Compound (3.1.2.6) was not active against bacteria *Bacillus megaterium* and *Escherichia coli*, but did exhibit activities toward fungi

Microbotryum violacea and *Mycotypha microspora*. Similarly, compound (3.1.2.6) exhibited activity against alga *Chlorella fusca* with a minimum inhibitory concentration of 7-11 μg, and toward brine shrimp *Artemia salina* at a 100% lethality rate using a test concentration at 0.5 mg/mL after 48 hours.



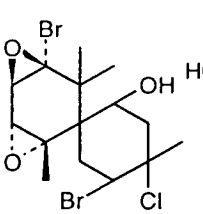
3.1.2.5



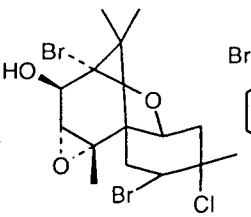
3.1.2.6

3.1.3 Polyhalogenated sesquiterpenes.

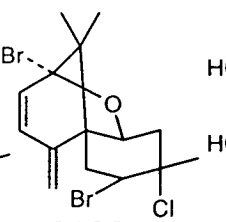
In 1996 four known halogenated sesquiterpenes, namely prepacifenol epoxide (3.1.3.1), johnstonol (3.1.3.2), pacifidiene (3.1.3.3), and the chamigrene diol compound (3.1.3.4) were reported from the sea hare *Aplysia dactylomela* by Pitombo *et al.*[15] The sample was collected from mid-intertidal rocks at Praia das Conchas-Cabo Frio, on the coast of Rio de Janeiro State, Brazil. The three frozen specimens of *Aplysia dactylomela* were carefully dissected into mantle and viscera. The acetone extract was partitioned between water and diethyl ether. The ether soluble material from the viscera extract, was further purified with silica gel and crystallization to afford the metabolites (3.1.3.1-3.1.3.4) while only metabolites (3.1.3.2 and 3.1.3.4) were isolated from the mantle and mucus. All metabolites were previously isolated from *Laurencia* and *Aplysia californica*.[15] The presence of two *Laurencia* species, *Laurencia obtusa* and *Laurencia flagellifera* at the same place where these animals were collected may be an explanation for the occurrence of these chamigrenes in *Aplysia dactylomela*.



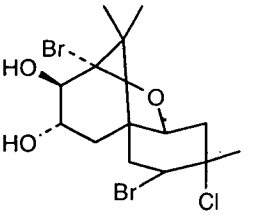
3.1.3.1



3.1.3.2



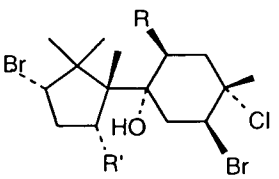
3.1.3.3



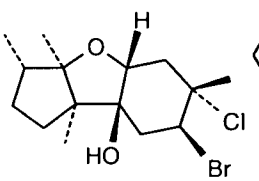
3.1.3.4

In 1999 McPhail *et al.*[16] reported four new (3.1.3.5-3.1.3.8) and two known sesquiterpenes, nidificene (3.1.3.9) and prepacifenol epoxide (3.1.3.1) from the digestive gland extracts of two colour variations of the sea hare *Aplysia dactylomela*. All four specimens, one of which was pale green and the other three were a shade of red, were

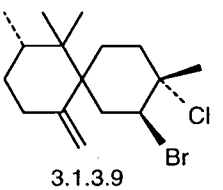
collected by hand from the intertidal zone at the same site in the Cape Recife Nature Reserve, Algoa Bay, on the Eastern Cape coast of South Africa. The acetone extracts of the two colour forms were separately partitioned between water and ethyl acetate. Purification of the ethyl acetate soluble material with silica gel column chromatography and crystallization gave compound 3.1.3.5 from the extracts of both colour forms. A further purification with HPLC of the more polar fractions afforded compounds (3.1.3.6) and (3.1.3.8) for the extract of the red colour form, while the green colour form extract gave compounds (3.1.3.1, 3.1.3.7, and 3.1.3.9). The structures were elucidated by spectroscopic methods, however, only compound (3.1.3.5) had its absolute stereochemistry established with a single-crystal X-ray diffraction experiment. Nidificene and prepacifenol epoxide were known *Laurencia* metabolites. The suspected red algal sources of these six compounds were unknown.[16]



3.1.3.5 R=OAc, R'=OH
3.1.3.6 R=H, R'=OH
3.1.3.7 R=R'=H

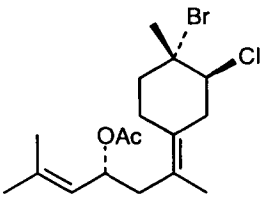


3.1.3.8

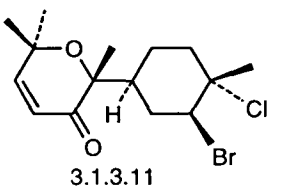


3.1.3.9

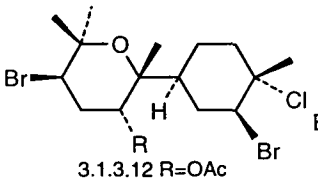
In 2000 three new sesquiterpenes, puertitol B acetate (3.1.3.10), caespitenone (3.1.3.11) and 8-acetylcaespitol (3.1.3.12) and nine known sesquiterpenes, caespitol (3.1.3.13), caespitane (3.1.3.14), laucapyranoid A (3.1.3.15), obtusol (3.1.3.16), cartilagineol (3.1.3.17), elatol (3.1.3.18), 9-isoobtusol (3.1.3.19), 9-acetylisoobtusol (3.1.3.20), and 9,15-dibromo-1,3(15)-chamigra-diene-11-ol (3.1.3.21) were also reported from *Aplysia dactylomela* by Wessels *et al.*[16] Three different samples of *Aplysia dactylomela* were collected from two locations of Tenerife, Canary Islands, Spain as one single animal from San Juan de la Rambla, one single animal from Punta del Hidalgo, and three animals from Punta del Hidalgo. Metabolites (3.1.3.5, 3.1.3.16-3.1.3.20) were isolated only from the San Juan de la Rambla specimens, while compounds (3.1.3.11, 3.1.3.21) were isolated from the Punta del Hidalgo extract. However, compounds (3.1.3.12-3.1.3.15) were isolated from all three different samples from both locations. Compounds (3.1.3.15-3.1.3.20, 3.1.3.22) were known metabolites from *Laurencia* sp.



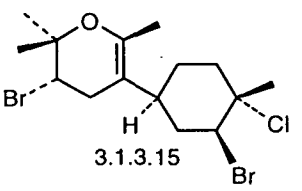
3.1.3.10



3.1.3.11

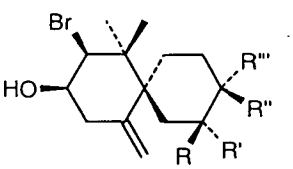


3.1.3.12 R=OAc
3.1.3.13 R=OH
3.1.3.14 R=H

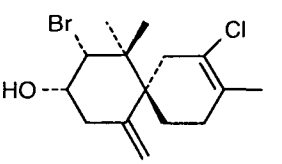


3.1.3.15

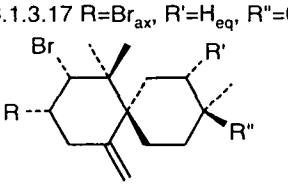
Compound (3.1.3.16) exhibited cytotoxicity at IC_{50} values of less than 1.0, 1.5 and 7.0 μg/mL toward cultures of liver, breast, and gastric cancer cell lines, respectively. Compound (3.1.3.18) exhibited cytotoxicity at IC_{50} values of less than 1.0 μg/mL toward all cultured gastric, liver, and breast cancer cell lines. Metabolite (3.1.3.21) showed IC_{50} values of 17 μg/mL toward cultured gastric cancer cell lines, and less than 1.0 μg/mL toward both cultured liver and breast cancer cell lines. The metabolites (3.1.3.18-3.1.3.21) were active against the bacterium *Bacillus megaterium*. Compounds (3.1.3.10, 3.1.3.16, 3.1.3.18, and 3.1.3.20-3.1.3.21) were active against fungi *Microbotryum violacea*, *Mycotypha microspora*, *Eurotium repens*, and *Fusarium oxysporum*. Compounds (3.1.3.13-3.1.3.14, and 3.1.3.16) were active against the alga *Chlorella fusca*. Moreover, compounds (3.1.3.13-3.1.3.14, 3.1.3.16, 3.1.3.18, and 3.1.3.21) were active toward brine shrimp *Artemia salina* at 70, 40, 80, and 100% lethality at a concentration 0.5 mg/mL, respectively.



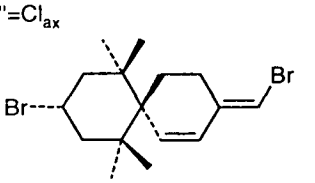
3.1.3.16 $R=H_{ax}$, $R'=Cl_{eq}$, $R''=Br_{eq}$, $R'''=CH_{3ax}$
3.1.3.17 $R=Br_{ax}$, $R'=H_{eq}$, $R''=CH_{3eq}$, $R'''=Cl_{ax}$



3.1.3.18



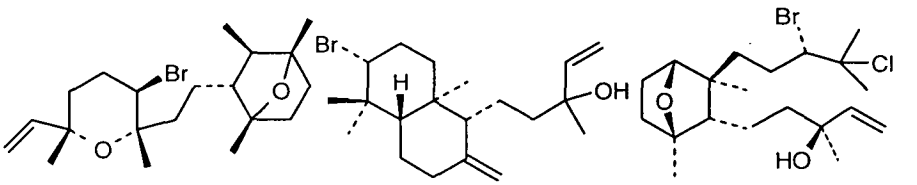
3.1.3.19 R=OH, $R'=Cl_{ax}$, $R''=Br_{ax}$
3.1.3.20 R=OAc, $R'=Cl_{ax}$, $R''=Br_{ax}$



3.1.3.21

3.1.4 Polyhalogenated diterpenes.

Recently Wessels *et al.*[14] reported two new diterpenes, dactylopyranoid (3.1.4.1) and isopinnatol B (3.1.4.2) and one known diterpene, dactylomelol (3.1.4.3) from the sea hare *Aplysia dactylomela* from two locations of Tenerife, Canary Islands, Spain. Dactylopyranoid (3.1.4.1) was isolated from three animals at Punta del Hidalgo, while isopinnatol B (3.1.4.2) and dactylomelol (3.1.4.3) were isolated from one single animal at San Juan de la Rambla. All three diterpenes were inactive against cultured cancer cell lines, bacteria, fungi, and algae. However, brine shrimp became hyperactive only when exposed to isopinnatol B (3.1.4.2) at 0.5 mg/mL after 48 hours.



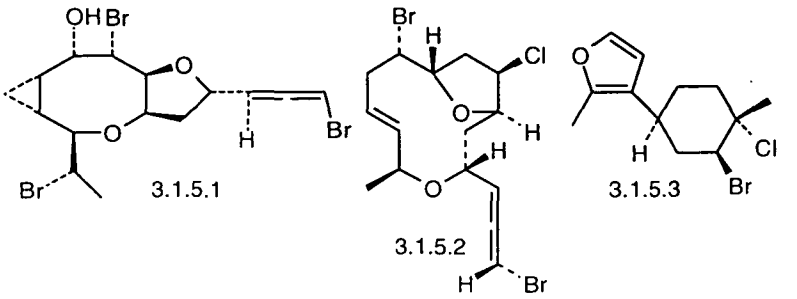
3.1.4.1 3.1.4.2 3.1.4.3

3.1.5 Nonterpenoid compounds.

In 1995 a new C_{15} acetogenin, namely aplyparvunin (3.1.5.1) was isolated from the sea hare *Aplysia parvula* by Miyamoto *et al.*[17] The sea hare was collected from Koinoura, Fukuoka Prefecture, Japan. The chloroform soluble portion of the chloroform-methanol extract was subjected to Sephadex LH-20 chromatography followed by silica gel column chromatography to afford five ichthyotoxic fractions. Compound (3.1.5.1) was purified by reverse phase MPLC. Its structure was elucidated with spectroscopic data and a single crystal X-ray analysis. The metabolite (3.1.5.1) showed ichthyotoxic activity against mosquito fishes at a lethal concentration (LC_{100}) of 3 ppm within 24 hours.

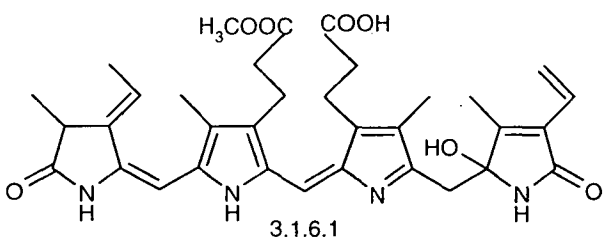
In 1997 Ciavatta *et al.*[18] reported a new C_{15} bromoallene, dactylallene (3.1.5.2) from the digestive gland of the sea hare *Aplysia dactylomela*, which was collected off the Canary Islands, Spain. The frozen specimens were dissected and the parapodia and digestive gland were separately extracted with acetone. Further purification of the ether soluble portion of the acetone extract of the digestive gland on silica gel column chromatography afforded the pure compound (3.1.5.2). X-ray diffractometric analysis determined its absolute stereochemistry. Dactylallene has a similar skeleton to obtusallene II which had been previously isolated from *Laurencia obtusa*. Test for the ichthyotoxicity of compound (3.1.5.2) against *Gambusia affinis* and its antifeedant activity against *Carassius auratus* were performed but no result has been shown in this publication.

A known metabolite, furocaespitane (3.1.5.3) was separated from the sea hare *Aplysia dactylomela* by Wessels *et al.*[16] Furocaespitane was isolated from both one single animal and three animals at Punta del Hidalgo, but it was not found in animals from San Juan de la Rambla, Tenerife, Canary Islands, Spain. This metabolite was previously reported from the red seaweed *Laurencia* sp. Furocaespitane was not cytotoxic and did not show activity in antibacterial, antifungal, antialgal, or brine shrimp assays.



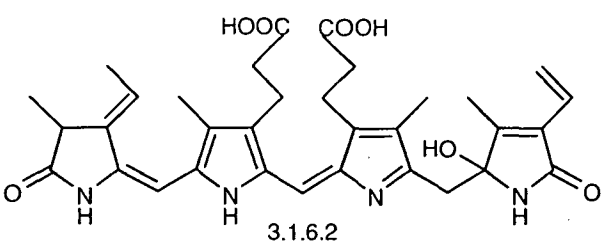
3.1.6 Tetrapyrrole compounds.

In 1966 aplysioviolin, which was isolated from the sea hare *Aplysia limacina* was assigned as structure (3.1.6.1).[19] UV and visible spectra, R_f from TLC and chemical degradation were used to determine its structure. Aplysioviolin was compared to urobilin, mesobiliviolin, mesobilirhodin, glaukobilin and mesobilipurpurin by R_f, as well as to bilipurpurin and mesobiliviolin by UV and visible absorption.[20]

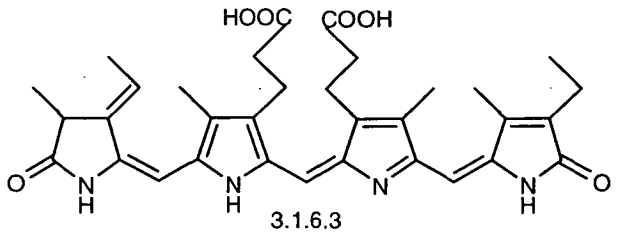


3.1.6.1

The author claimed that a purple pigment (3.1.6.2) was an unesterified form of aplysioviolin (3.1.6.1).[21] The purple pigment gave a transient deep blue colour in alkali, which was also a characteristic of aplysioviolin. A blue pigment was proposed as phycobilin 630 (3.1.6.3).

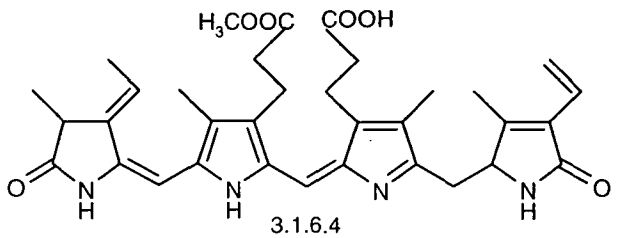


3.1.6.2



3.1.6.3

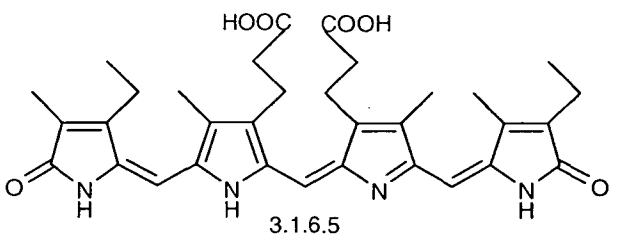
In 1967 a revised structure of aplysioviolin (3.1.6.4) was proposed.[22] Structure (3.1.6.4) has been used for aplysioviolin since then.



3.1.6.4

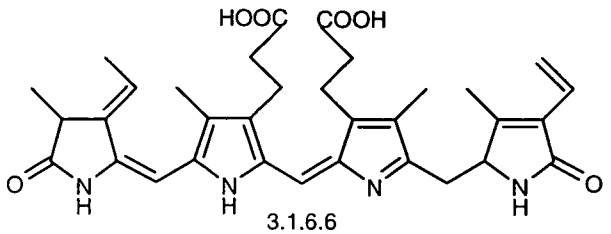
C-Phycocyanin is a photosynthetically active algal protein and has a tetrapyrrolic chromophore, called phycocyanobilin, which was previously mentioned as phycobilin 630 (3.1.6.3). It was isolated from cyanobacterium *Phormidium luridum* and from cyanobacterium *Synechococcus lividus*.[23] The structure of phycocyanobilin (3.1.6.3) was proposed based on ^{1}H NMR spectroscopy.

Phycocyanobilin (3.1.6.3) is an isomer of mesobiliverdin (3.1.6.5). ^{1}H NMR data of phycocyanobilin dimethyl ester and mesobiliverdin dimethyl ester were reported.[24]



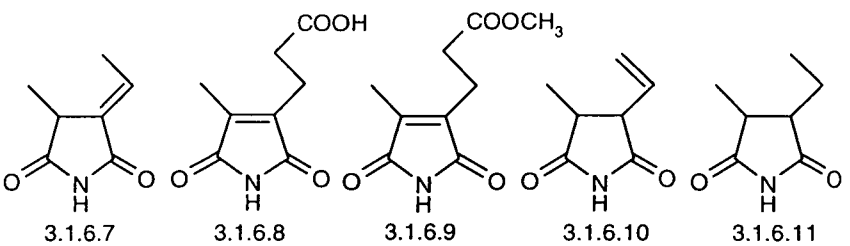
3.1.6.5

Similarly, C-phycoerythrin is a photosynthetically active red biliprotein from blue-green algae. It has a tetrapyrrole chromophore, phycoerythrobilin (3.1.6.6), which is isomeric with phycocyanobilin (3.1.6.3). Phycoerythrobilin (3.1.6.6) was prepared from *Phormidium persicinum* cells. The phycoerythrobilin free acid was esterified with diazomethane to give phycoerythrobilin dimethyl ester. ^{1}H NMR data of phycoerythrobilin dimethyl ester was reported. UV and visible absorption spectrum of phycoerythrobilin dimethyl ester was observed and similar to that of mesobilirhodin dimethyl ester and mesobiliviolin dimethyl ester.[25]



3.1.6.6

Both phycoerythrins and phycocyanins are photosynthetically active algal biliproteins. They have bile pigment prosthetic groups, called phycobilins. The phycobilins are attached to the protein components through covalent bonds. R-Phycoerythrin was from *Rhodymenia palmate*. The phycoerythrin was isolated and purified by repeated fractionation and crystallization from ammonium sulfate solutions at pH 7 and 4.5. Phycoerythrobilin (from R-phycoerythrin) and phycobilin-630 (from C-phycocyanin) were isolated, following hydrolysis of the biliproteins. Microtechniques for an oxidative degradation of bilins and biliproteins were used. Typical thin-layer chromatograms of imides derived from phycobilins and biliproteins by oxidative degradation (3.1.6.7-3.1.6.11) as well as purple pigment, blue pigment, R-phycoerythrin, C-phycocyanin were also investigated. Moreover, thin-layer chromatography of the dimethyl esters of aplysioviolin, purple pigment, and authentic bile pigments (mesobiliviolin, mesobilirhodin, mesobiliverdin, biliverdin, and I-urobilin) were studied. UV and visible spectral data of aplysioviolin dimethyl ester and purple pigment dimethyl ester were reported.[26] The authors indicated that the purple pigment was the dicarboxylic acid form of aplysioviolin and aplysioviolin was the monomethyl ester of phycoerythrobilin as previously described.

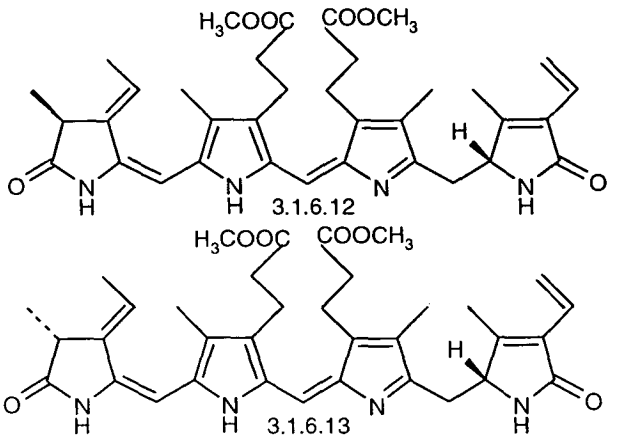


3.1.6.7 3.1.6.8 3.1.6.9 3.1.6.10 3.1.6.11

A sequence analysis on the microscale (0.5 μg pigment) by the degradation of bile pigments with chromic acid and chromate was investigated. These pigments were aplysioviolin, mesobiliviolin, biliverdin, mesobiliverdin, and urobilin.[27]

Bile pigments from several invertebrates, including the sea hares (*Aplysia depilans*, *Aplysia punctata*, *Aplysia limacine*, *Aplysia californica*) were studied. Aplysioviolin (3.1.6.4), aplysioverdin, and aplysiourobilin were from the sea hares *Aplysia*.[28]

A mixture of two diastereomeric bile pigments, compound (3.1.6.12) and its enantiomer as well as compound (3.1.6.13) and its enantiomer were synthesised. After separation of the two components by preparative thin-layer chromatography, the spectral properties of one of them, racemic (3.1.6.13) was identical with those of phycoerythrobilin dimethyl ester.[29]



3.2 General introduction and secondary metabolites from *Laurencia*.

The red seaweed genus *Laurencia* is common worldwide. *Laurencia filiformis* (C. Agardh) Montagne 1845 in this study belongs to phylum Rhodophyta, class Ceramiales, and family Rhodomelaceae. This species is found in sheltered and calm waters.[30]

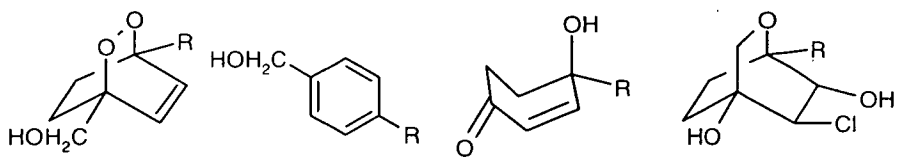
In 1973 Scheuer[9] reported sesquiterpenes, diterpenes, and cyclic ethers from *Laurencia*. Then five years later Martin *et al.*[31] reviewed algal sesquiterpenes from *Laurencia* in five categories of compounds, monocyclofarnesanes, bisabolanes, chamigranes, and other skeletons. In 1978 algal nonisoprenoids, acetylenes and a related aldehyde from *Laurencia* were reported by Moore.[32] In 1981 Howard *et al.*[33] reviewed the structures and proposed biosynthesis of regular terpenoids and rearranged terpenoids *from Laurencia*. Two years later Erickson[34] reported constituents of *Laurencia* into five structural types, sesquiterpenoids, diterpenoids, triterpenoids, C_{15} acetogenins, and miscellaneous compounds. Ecology and physiological activity were also mentioned.

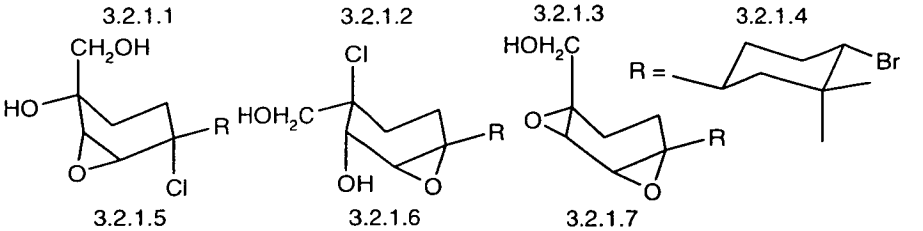
This review will discuss all secondary metabolites discovered in the red seaweed genus *Laurencia* covering the literature published from 1995 to 2000 by SciFinder Scholar searching, focusing on methods of isolation, structure determination and biological activity where possible. Secondary metabolites will be discussed in compound types.

3.2.1 Sesquiterpenes.

In 1995 Erickson *et al.*[35] isolated seven new sesquiterpenes, majapolene A (3.2.1.1), majapolene B (3.2.1.2), majapolone (3.2.1.3), majapol A (3.2.1.4), majapol B (3.2.1.5), majapol C (3.2.1.6), and majapol D (3.2.1.7) from *Laurencia majuscula*. The alga was collected from Apo Island, near the southern tip of Negros Island, Central Visayas, Philippines. The methanol-chloroform crude extract was partitioned between 90% aqueous methanol and hexane and further extracted with tetrachloromethane. The tetrachloromethane soluble material was subjected to Sephadex LH-20, silica gel column to give the metabolites (3.2.1.1-3.2.1.7). Majapolene A (3.2.1.1) showed modest activity in the NCI 60-cell line cytotoxicity screen.

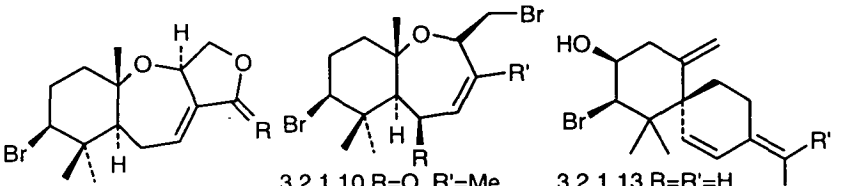
Five known sesquiterpenes, aplysistatin (3.2.1.8), palisadin A (3.2.1.9), palisadin B (3.2.1.10), 5-acetoxypalisadin B (3.2.1.11), 12-hydroxypalisadin B (3.2.1.12) were reported from *Laurencia karlae* by Su *et al.*[36] The seaweed was collected from the Nansha Islands in the South China Sea. The sun-dried specimens were extracted with ethyl alcohol and partitioned between ethyl acetate and water. The ethyl acetate soluble portion was subjected to vacuum liquid chromatography over silica gel H. Further purification by preparative TLC and flash chromatography on silica gel H with 10% diethyl ether in petrol, as well as recrystallization from acetone, gave the metabolites (3.2.1.8-3.2.1.12).





In 1995 Rashid *et al.*[37] reported three known brominated chamigrane sesquiterpenes (3.2.1.13-3.2.1.15) from *Laurencia majuscula*, which was collected along the coast of northeastern Australia. The frozen alga was ground and extracted with water, lyophilized and further extracted with dichloromethane-methanol (1:1) and 100% methanol. The combined organic extracts were partitioned and the antitumour activity in the hexane soluble fraction was separated by vacuum-liquid chromatography on diol bonded-phase. The active portions were resolved by amino bonded-phase HPLC with hexane-isopropanol

(49:1) to give compounds (3.2.1.13-3.2.1.14) and by silica gel HPLC with hexane-isopropanol (99:1) to give compound (3.2.1.15). These metabolites were cytotoxic against the National Cancer Institute's *in vitro* primary antitumour screening assay.



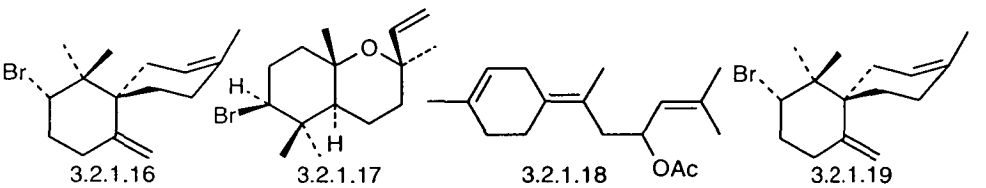
3.2.1.8 R=O
3.2.1.9 R=H_2

3.2.1.10 R=O, R'=Me
3.2.1.11 R=OAc, R'=Me
3.2.1.12 R=H, R'=CH_2OH

3.2.1.13 R=R'=H
3.2.1.14 R=Br, R'=H
3.2.1.15 R=H, R'=Br

A new β-chamigrene, (+)-(10*S*)-10-bromo-β-chamigrene (3.2.1.16) was isolated from *Laurncia rigida* by Konig *et al.*[38], collecting in Australia. This report used both two dimensional nuclear magnetic resonance pulsed field gradient spectroscopy (PFGS) and conventional phase cycled method to determine the structure of (3.2.1.16). No details of its isolation were reported in this publication.

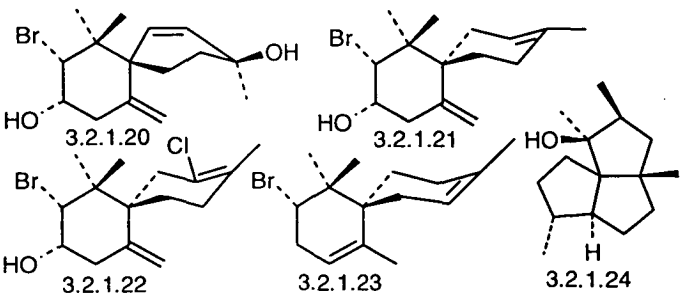
In 1997 Suzuki *et al.*[39] reported a known halogenated sesquiterpene, 3-β-bromo-8-epicaparrapioxide (3.2.1.17) from *Laurencia obtusa*, which was collected at Scanlon's Island, the western coast of Ireland. The dried alga was extracted with methanol and partioned with ether and water. The ether soluble portion was subjected to silica gel column chromatography and further purified on a thin-layer plate with hexane-ethyl acetate (9:1) to give (3.2.1.17).



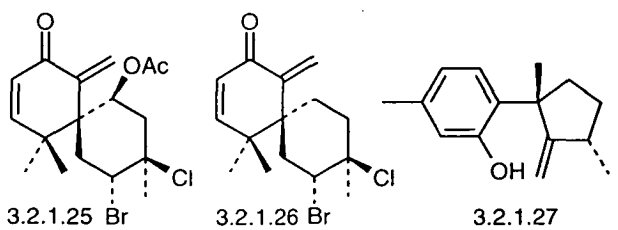
3.2.1.16 3.2.1.17 3.2.1.18 3.2.1.19

Four new sesquiterpenes, (+)-(10*S*)-10-bromo-β-chamigrene (3.2.1.16), 3-acetoxy-*E*-γ-bisabolene (3.2.1.18), (-)-10α-bromo-9β-hydroxy-α-chamigrene (3.2.1.19), and rigidol (3.2.1.20), together with four known sesquiterpenes (3.2.1.21-3.2.1.24) were reported from *Laurencia rigida* by Konig *et al.*[40] These known metabolites were deschloroelatol (3.2.1.21), elatol (3.2.1.22), (-)-(10*R*)-10-bromo-α-chamigrene (3.2.1.23), and (3.2.1.24). The alga was collected at Cape Banks, Sydney, Australia. The dried alga was extracted with dichloromethane and methanol. The dichloromethane soluble material was subjected to vacuum liquid chromatography over silica gel and further purified with normal phase HPLC to afford these metabolites. Metabolites (3.2.1.18-3.2.1.19, 3.2.1.21, and 3.2.1.24)

showed moderate antialgal activity against *Chlorella fusca*. Compounds (3.2.1.19-3.2.1.20, and 3.2.1.21-3.2.1.22) showed moderate antifungal properties against *Mycotypha microspora* and *Eurotium repens*.



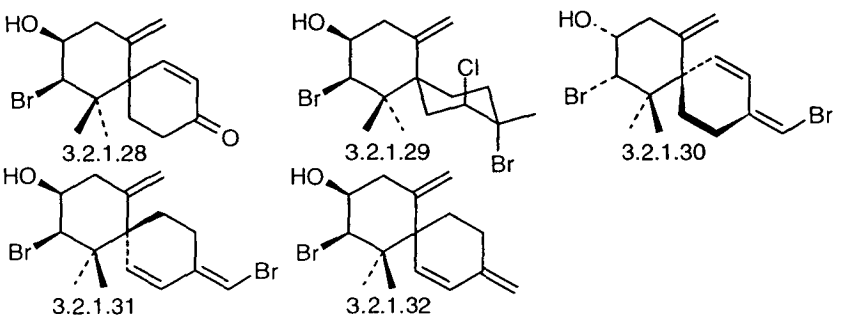
In 1997 Masuda *et al.*[41] reported two chamigrane-type sesquiterpenoids, (3.2.1.25-3.2.1.26) and a laurane-type sesquiterpenoid (3.2.1.27) from *Laurencia majuscula*. (2*R*, 3*R*, 5*S*)-5-Acetoxy-2-bromo-3-chlorochamigra-7 (14), 9-dien-8-one (3.2.1.25), (2*R*, 3*R*)-2-bromo-3-chlorochamigra-7 (14), 9-dien-8-one (3.2.1.26), and debromoisolaurinterol (3.2.1.27) were separated from this red seaweed. The specimens for morphological and chemical studies were collected at Taketomi Island and Hateruma Island, the Ryukyu Islands, Japan. Living specimens were used for morphological study, however, material for chemical analysis was air-dried at room temperature for a day and the methanol extract was partitioned between ether and water. The organic soluble fraction was subjected to silica gel column chromatography and repeated preparative thin-layer chromatography with hexane-dichloromethane (7:3) to give these three metabolites (3.2.1.25-3.2.1.27). The results from both places were compared by TLC. The methanol extracts from Huteruma Island showed identical TLC profiles to those of Taketomi Island extracts.



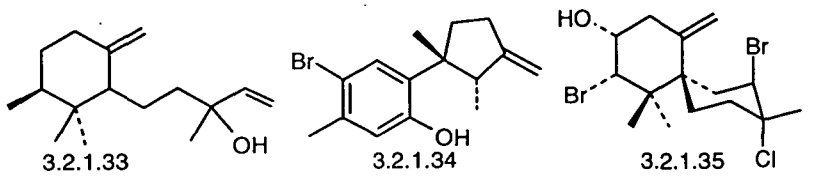
Two new chamigranes (3.2.1.28-3.2.1.29) and four known sesquiterpenes (3.2.1.22, 3.2.1.30-3.2.1.32) were reported from *Laurencia cartilaginea* by Juagdan *et al.*[42] The sample was collected at Ma'ili Pt. Park off the Wai'anae coast of O'ahu, Hawaii. Ma'ilione or 8-bromo-9-hydroxychamigra-2(3),11(12)-dien-1-one (3.2.1.28) was a new norsesquiterpene. Metabolite (3.2.1.29) is a diastereoisomer of isoobtusol, namely *allo*-isoobtusol, or 2-chloro-1, 8-dibromochamigr-11(12)-en-9-ol. The known metabolites were

elatol (3.2.1.22), [1 (15)*Z*, 2*Z*, 4*R*, 8*S*, 9*R*]-8, 15-dibromochaigra-1(15), 2, 11(12)-trien-9-ol (3.2.1.30), [1(15)*E*, 2*Z*, 4*R*, 8*S*, 9*R*]-8,15-dibromochamigra-1(15), 2, 11(12)-trien-9-ol (3.2.1.31), and isoobtusadiene (3.2.1.32). The wet specimen was extracted with methanol and separated by high-speed counter-current chromatography guided by P388, A549, HT29 and MEL28 assays. The active fractions were subjected to silica gel HPLC with hexane-ethyl acetate (8:2), then hexane-ethyl acetate (85:15) to give these metabolites (3.2.1.22, 3.2.1.28-3.2.1.32).

In 1997 Imre *et al.*[43] investigated *Laurencia obtusa*, which was collected from three different sites of the Aegean coast, Turkey. β-Synderol (3.2.1.33) was separated only from a collection of Didim near Aydin, Aegean coast. Neither the site at Assos near Canakkale nor the site at Guvercinlik near Bodrum contained β-synderol (3.2.1.33). The air-dried samples were extracted with chloroform-methanol (2:1) and chromatographed on a silica gel column. Further purification with normal phase HPLC was carried out to give (3.2.1.33). β-Synderol (3.2.1.33) showed an activity towards brine shrimp *Artemia salina* at LC_{50} of 35.1 mg/L.



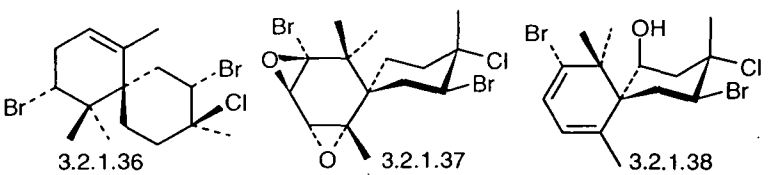
A known sesquiterpene, allolaurinterol (3.2.1.34) was reported from *Laurencia obtusa* by Konig *et al.*[44] The alga was collected from the reef top in front of the Lauro Club, Salisbury, Caribbean Island of Dominica. The freeze-dried alga was extracted with dichloromethane and showed significant antibacterial activity. Vacuum liquid chromatography over silica gel and LiChrosorb Si60 HPLC were used to purify allolaurinterol. The metabolite showed moderate antifungal activity towards the fungus *Ustilago violacea*, and antibacterial property against Gram-positive bacteria *Bacillus megaterium*.



3.2.1.33 3.2.1.34 3.2.1.35

In 1998 Francisco *et al.*[45] revised the structure of the formerly *allo*-isoobtusol (3.2.1.29) to cartilagineol (3.2.1.35). The assignments for the chlorine and bromine carbons were reversed between the previous structure and the new one. The carbon chemical shift values and HMQC spectrum confirmed that the quaternary halide at C-9, 73.3 ppm, should be assigned as chlorine and the methine halide at C-8, 57.1 ppm, should be assigned as bromine. Further evidence for both the regiochemistry and stereochemistry of cartilagineol was shown by an X-ray crystal structure.

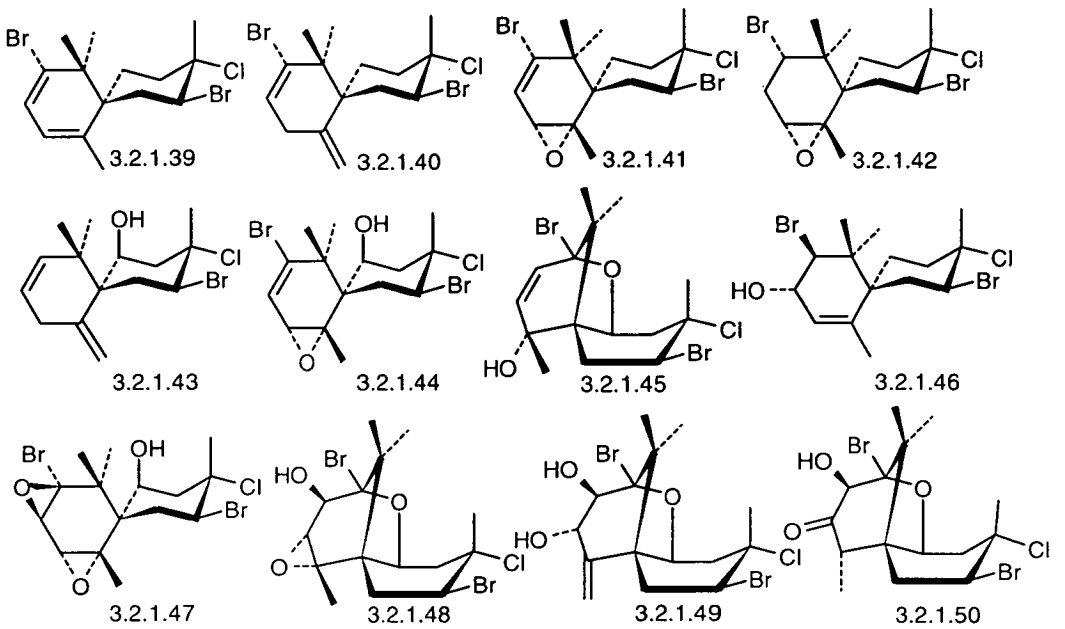
A new sesquiterpene, 2,10-dibromo-3-chloro-α-chamigrene (3.2.1.36) was reported from *Laurencia japonensis* by Takahashi *et al.*[46] Six samples were collected from different locations at Chinzei, saga Prefecture, Heki, Yamaguchi Perfecture, Mihonoseki, Shimane Prefecture, Iwami, Tottori Prefecture, Toyooka, Hyogo Prefecture, and Shimoda, Shizuoka Prefecture, Japan. All six samples contained the metabolite (3.2.1.36) in different yields. The dried alga was extracted with methanol and partitioned between ether and water. The ether soluble fraction was purified with silica gel column chromatography and preparative TLC to give compound (3.2.1.36).



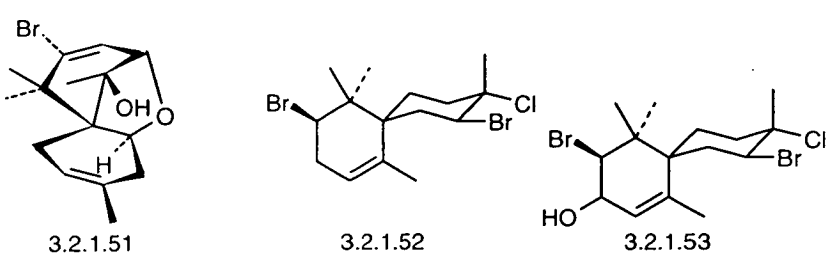
3.2.1.36 3.2.1.37 3.2.1.38

In 1999 Kimura *et al.*[47] reported two new sesquiterpenes (3.2.1.37-3.2.1.38), ten known sesquiterpenes (3.2.1.39-3.2.1.48), and two artifacts (3.2.1.49-3.2.1.50) from *Laurencia nidifica*. The alga was collected at Pupukea, Oahu. The freeze-dried sample was extracted with methanol and partitioned between chloroform and water. The chloroform soluble fraction was subjected to silica gel flash chromatography and normal phase HPLC to give the metabolites, 2,10-dibromo-3-chloro-7,8:9,10-diepoxychamigrane (3.2.1.37), 2,10-dibromo-3-chlorochamigrane-7,9-dien-5-ol (3.2.1.38), 2,10-dibromo-3-chloro-α-chamigrene (3.2.1.39), nidificene (3.2.1.40), deoxyprepacifenol (3.2.1.41), 2,10-dibromo-3-chloro-7,8-epoxychamigrane (3.2.1.42), nidifidienol (3.2.1.43), prepacifenol (3.2.1.44), pacifenol (3.2.1.45), 2,10-dibromo-3-chlorochamigran-7-en-9-ol (3.2.1.46), prepacifenol

epoxide (3.2.1.47), johnstonol (3.2.1.48). Nidificene (3.2.1.40) and nidifidienol (3.2.1.43) showed good antiviral activity against HSV-1.



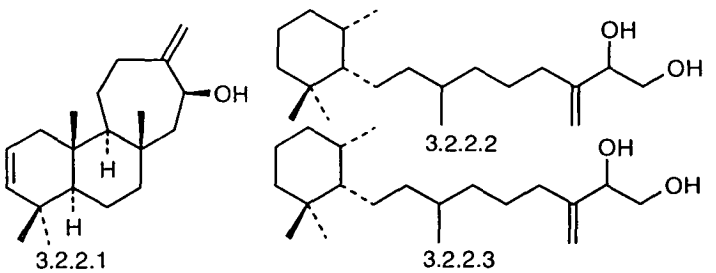
A new sesquiterpene, claviol (3.2.1.51), and five known sesquiterpenes, deoxyprepacifenol (3.2.1.41), prepacifenol (3.2.1.44), pacifenol (3.2.1.45), 4,10-dibromo-3-chloro-α-chamigrene (3.2.1.52), and 9-hydroxy-4,10-dibromo-3-chloro-α-chamigrene (3.2.1.53) were reported from *Laurencia claviformis* by Rovirosa *et al.*[48] The alga was collected at Vaihu Easter Island, Chile. All six metabolites were tested for their effect on the inhibition of cytokinesis in the sea urchin *Tetrapygus niger* embryos. The compound (3.2.1.53) was more active than others with an ED_{50} value of 22.7 μg/mL. The new compound (3.2.1.51) showed the ED_{50} value of 45.2 μg/mL. However, the activity of compound (3.2.1.53) could be considered mild compared with other active marine compounds as stypoldione, which showed the ED_{50} value of 1.1 μg/mL.



3.2.2 Diterpenes.

In 1995 Su *et al.*[38] reported a new diterpene, laukarlaol (3.2.2.1) and five known sesquiterpenes (3.2.1.8-3.2.1.12) from *Laurencia karlae*. The ethyl acetate soluble portion was subjected to vacuum liquid chromatography over silica gel H and further purified with silica gel preparative TLC with 10% ether-petrol to give (3.2.2.1).

In 1996 two new cyclophytane-type diterpenes, viridiol A (3.2.2.2) and viridiol B (3.2.2.3) were reported from *Laurencia viridis* by Norte *et al.*[49] The alga was collected in the intertidal zone at Callao Salvaje, Tenerife, Canary Islands, Spain. The air-dried alga was extracted with chloroform-methanol (1:1) and chromatographed on a silica gel column. A further purification was done on Sephadex LH-20 using *n*-hexane-chloroform-methanol (2:1:1). A medium pressure silica gel chromatography using *n*-hexane-ethyl acetate (7:3) and a medium pressure reverse phase using methanol-water (9:1) were carried out. Finally, HPLC using a μ-Bondapak C-18 column with acetonitrile-water (9:1) and μ-Porasil HPLC column using *n*-hexane-ethyl acetate (7:3) and *n*-hexane-ethyl acetate (3:2) were performed to afford pure viridiol A (3.2.2.2) and viridiol B (3.2.2.3), respectively. Viridiol A showed cytotoxicity against cell lines P388 suspension culture of a lymphoid neoplasm from a mouse, A549 monolayer culture of a human lung carcinoma, HT29 monolayer culture of a human colon carcinoma, MEL-28 monolayer culture of a human melanoma, protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis at IC_{50} of 1, 2.5, 2.5, 2.5, 10, 10 and 3 μg/mL, respectively. Similarly, viridiol B showed cytotoxicity against cell lines P388, A549, HT29, MEL28, protein, DNA and RNA at IC_{50} of 1, 2.5, 2.5, 2.5, >10, 10 and 8 μg/mL, respectively.



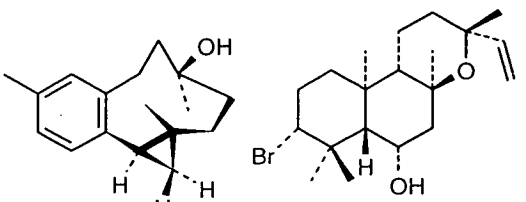
3.2.2.1 3.2.2.2 3.2.2.3

In 1996 Zeng *et al.*[50] reported a new diterpene, benkarlaol (3.2.2.4) from *Laurencia karla*, which was collected from the Nansha Islands in the South China Sea. The sun-dried alga was extracted with ethanol and partitioned between ethyl acetate and water. Vacuum liquid chromatography and preparative TLC on Si gel were used for purification to give

benkarlaol. Basic ^{1}H, ^{13}C NMR, and 2D HMQC, HMBC, COSY and NOESY experiments were used to assign the structure and relative stereochemistry for (3.2.2.4).

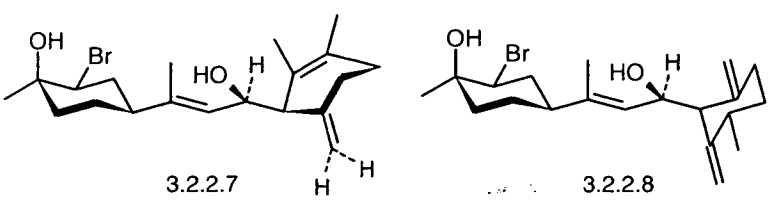
In 1997 a new *ent*-labdane bromoditerpene, (-)-paniculatol (3.2.2.5) was reported from *Laurencia paniculata* by Briand *et al.*[51] The sample was collected at Al Wakrah Bay, south of Doha, Qatar, Arabian Gulf. The air-dried alga was extracted with methanol-chloroform (1:1) and partitioned between ether and water. The organic fraction was rechromatographed on silica gel, further purified with preparative TLC and recrystallization in hexane to give the metabolite (3.2.2.5). This structure contained a tetrahydropyran ring as shown by basic NMR. The absolute configuration was determined by X-ray crystallography.

In 1997 Guella *et al.*[52] also reported a new obtusane diterpene, rogioldiol A (3.2.2.6) from *Laurencia microcladia*, which was collected from Il Rogiolo along the Coast of Tuscany. Reverse phase HPLC with acetonitrile-water (65:35), followed by cyano HPLC with hexane-isopropanol (97:3) and finally Si60 HPLC with hexane-ethyl acetate (4:1) were used to give (3.2.2.6).



3.2.2.4 3.2.2.5 3.2.2.6

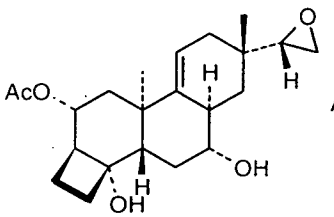
In 1998 two new obtusane diterpenes, rogioldiol B (3.2.2.7) and rogioldiol C (3.2.2.8) were also reported from *Laurencia microcladia* by Guella *et al.*[53] The specimen was collected at Il Rogiolo along the coast of Tuscany, Italy. Reverse phase HPLC with acetonitrile-water (65:35), followed by cyano HPLC with hexane-isopropanol (97:3) and finally Si60 HPLC with hexane-ethyl acetate (1:1) were used to give (3.2.2.7-3.2.2.8). The absolute configurations were studied using Mosher's ester method, exciton-coupling techniques, NOE experiments, and molecular mechanics calculations.



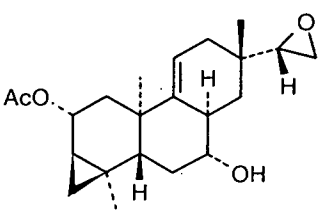
3.2.2.7 3.2.2.8

In 1998 Kurata *et al.*[54] reported three new diterpenes (3.2.2.9-3.2.2.11), thirteen known diterpenes (3.2.2.12-3.2.217, 3.2.2.19-3.2.2.25) from *Laurencia saitoi*, which was

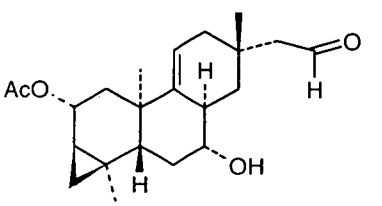
collected at Suttsu near Iwanai, western Hokkaido, Japan. Furthermore, parguerol 7-acetate (3.2.2.13) and parguerol 19-acetate (3.2.2.15) gave parguerol triacetate (3.2.2.18) on acetylation with acetic anhydride and pyridine. The dried alga was extracted with methanol and partitioned between ether and water. The ethereal solution was shaken with 5% KOH, washed with water to give a neutral oil which showed potent feeding-deterrent activity. The neutral fraction was subjected to column chromatography over alumina. Further purification using reverse phase and normal phase HPLC, and preparative TLC were performed to afford these metabolites. The structures were established with basic ^{1}H, ^{13}C NMR and 2D HMBC, COSY and HSQC. The relative configuration was determined by NOE spectroscopy. Compounds (3.2.2.18, 3.2.2.23, and 3.2.2.24) showed potent feeding-deterrent activity against the young abalone *Haliotis discus hannai*, while other compounds showed moderate or weak activity. Moreover, compounds (3.2.2.18 and 3.2.2.23) showed potent feeding-deterrent activity against the young sea urchins *Stronglyocentrotus nudus* and *Stronglyocentrotus intermedius*.



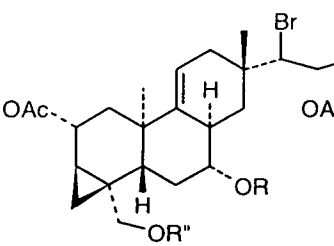
3.2.2.9



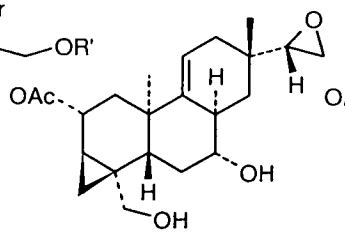
3.2.2.10



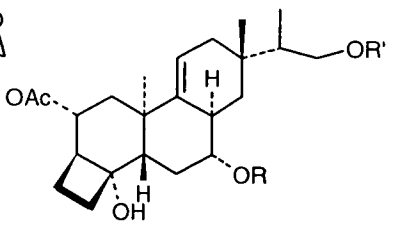
3.2.2.11



3.2.2.12 R=R'=R"=H
3.2.2.13 R=Ac, R'=R"=H
3.2.2.14 R=R"=H, R'=Ac
3.2.2.15 R=R'=H, R"=Ac
3.2.2.16 R=R'=Ac, R"=H
3.2.2.17 R=H, R'=R"=Ac
3.2.2.18 R=R'=R"=Ac



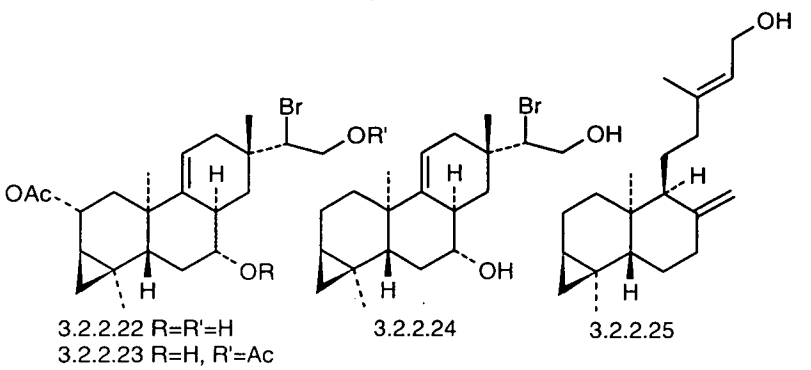
3.2.2.19

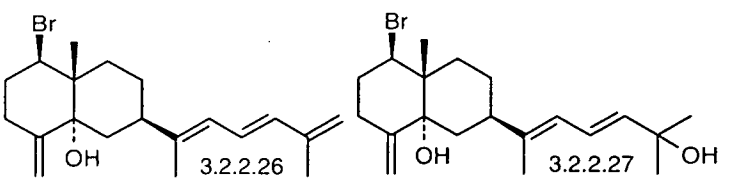


3.2.2.20 R=R'=H
3.2.2.21 R=R'=Ac

In 1998 Takahashi *et al.*[48] also reported a new diterpene, anhydroaplysiadiol (3.2.2.26) and a known diterpene, aplysiadiol (3.2.2.27) from *Laurencia japonensis*. Six samples were collected from different locations at Chinzei, saga Prefecture, Heki, Yamaguchi Perfecture, Mihonoseki, Shimane Prefecture, Iwami, Tottori Prefecture, Toyooka, Hyogo Prefecture, and Shimoda, Shizuoka Prefecture. Only the Chinzei sample

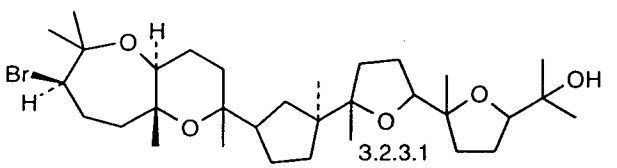
contained anhydroaplysiadiol (3.2.2.26). However, all six samples contained aplysiadiol (3.2.2.27) in different yields. The dried alga was extracted with methanol and partitioned between ether and water. The ether soluble fraction was purified with silica gel column chromatography and preparative TLC to give the metabolites (3.2.1.36, 3.2.2.26, and 3.2.2.27).



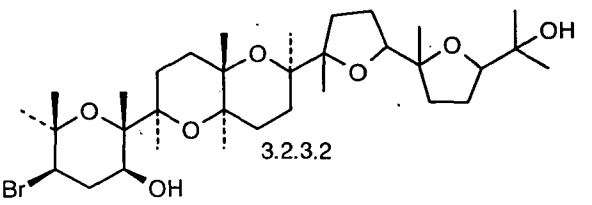


3.2.3 Triterpenes

In 1995 Matsuo *et al.*[55] reported a new squalene-derived pentacyclic triterpene alcohol, enshuol (3.2.3.1) from *Laurencia omaezakiana*, which was collected at Omaezaki, Shizuoka Prefecture on the Pacific coast of central Japan. The structure was determined by basic ^{1}H, ^{13}C NMR, and 2D NMR, COSY, HOHAHA, HMBC, NOESY and HSQC experiments.

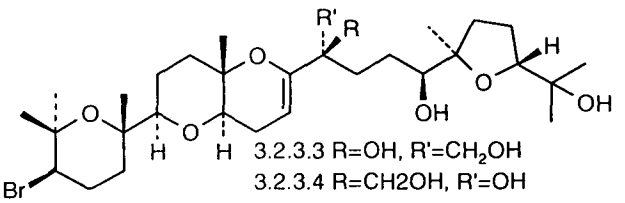


In 1995 Suzuki *et al.*[56] reported a new bromotriterpene polyether, callicladol (3.2.3.2) from *Laurencia calliclada*, which was collected at An Thoi, Phu Quoc Island, Kien Giang Province, Vietnam. Its structure was determined using basic ^{1}H, ^{13}C NMR, and 2D NMR, COSY, HOHAHA, NOESY and HSQC experiments.



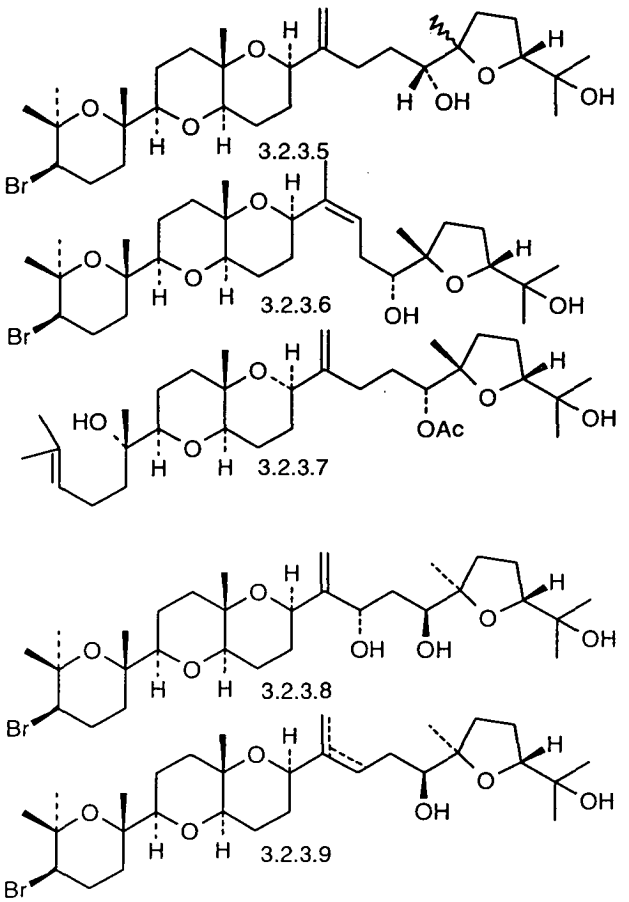
3.2.3.2

In 1997 Norte *et al.*[57] reported two new polyether triterpenes, thyrsenol A (3.2.3.3), and thyrsenol B (3.2.3.4) from *Laurencia viridis*. The alga was collected near Callao Salvaje, Tenerife, Spain. The dried alga was extracted with chloroform-methanol (1:1). The crude extract was purified by silica gel medium pressure and Sephadex LH-20 column chromatography, and μ-Porasil HPLC to give pure thyrsenol A and thyrsenol B. The structures were established by using COSY, HMQC, HMQC-TOCSY, and HMBC experiments. The relative stereochemistry was determined on the basis of ROESY and NOE difference data. Thyrsenol B (3.2.3.4) showed a potent activity (IC_{50} = 0.01 μg/mL, 0.016 μM) significantly higher than thyrsenol A (3.2.3.3) (IC_{50} = 0.25 μg/mL, 0.40 μM) against cultured tumour cells line P388.



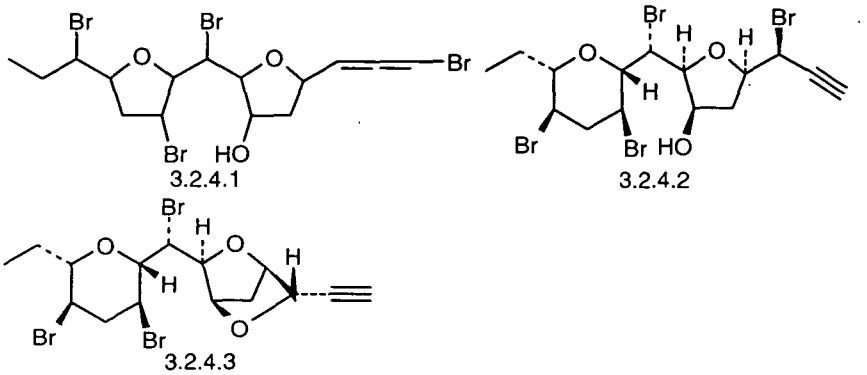
3.2.3.3 R=OH, R'=CH_2OH
3.2.3.4 R=CH2OH, R'=OH

In 1997 Norte *et al.*[58] reported five new polyether triterpenes with a squalene skeleton, dehydrovenustatriol (3.2.3.5), 15,16-dehydrovenustatriol (3.2.3.6), predehydro-venustatriol acetate (3.2.3.7), 16-hydroxydehydrothyrsiferol (3.2.3.8) and 10-epi-15,16-dehydrothyrsiferol (3.2.3.9) from *Laurencia viridis*. The alga was collected in the intertidal zone at Callao Salvaje, Paraiso Floral, El Palmar, Tenerife, Canary Islands, Spain. The dried alga was extracted with dichloromethane in a Soxhlet apparatus for 24 hours and with dichloromethane-methanol (1:1) at room temperature. The extracts were combined and subjected to silica gel column, Sephadex LH-20, medium pressure silica gel, medium pressure reverse phase chromatography and HPLC to afford these metabolites. ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC, and ROESY experiments were performed to establish the structures.



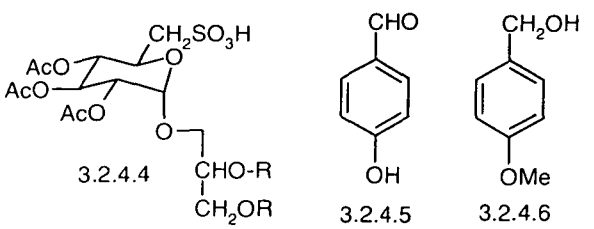
3.2.4 Nonterpenoids

In 1995 Imre *et al.*[59] reported a new polybrominated bicyclic ether with a bromoallenic side chain (3.2.4.1) from *Laurencia obtusa* and two known polybrominated C_{15} acetogenins (3.2.4.2-3.2.4.3) from *Laurencia paniculata*. The specimens were collected at Cesmealti near Izmir, and at Guvercinlik near Bodrum, Turkey. The air-dried alga *Laurencia paniculata* was extracted with ether in a Soxhlet apparatus, while the air-dried alga *Laurencia obtusa* was macerated with chloroform-methanol (2:1). Both extracts were subjected to silica gel columns twice and further recrystallization from hexane-ether to give these metabolites. As attempts to obtain suitable crystals of compound (3.2.4.1) for X-ray analysis were failed, only its planar structure could be proposed.

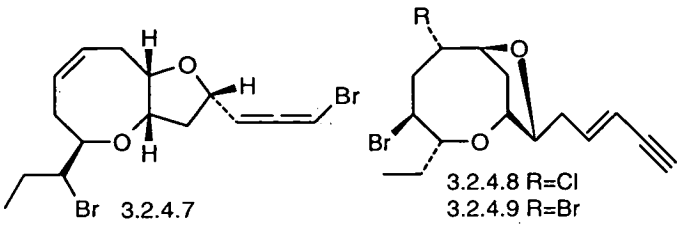


In 1995 Siddhanta *et al.*[60] reported a sulfonoglycolipid (3.2.4.4) from the methanol extract of *Laurencia pedicularioides*, which was collected from Veraval on the west coast of India.

In 1996 Wright *et al.*[61] reported two known aromatic compounds, *p*-hydroxy-benzaldehyde (3.2.4.5) and *p*-methoxybenzyl alcohol (3.2.4.6) from *Laurencia papillosa*. The alga was collected from the coast of the Caribbean Island of Dominica. The freeze-dried sample was extracted with dichloromethane and then methanol. The dichloromethane soluble portions were chromatographed over silica gel and further purified with normal phase HPLC with acetone-hexane (3:17) to give these compounds. Both compounds showed weak *in vitro* antimalarial activity.



In 1996 Suzuki *et al.*[62] reported a new C_{15} nonterpenoid bromoallene, pannosallene (3.2.4.7) and two known C_{15} nonterpenoids, chlorofucin (3.2.4.8) and bromofucin (3.2.4.9) from *Laurencia pannosa*, which was collected at An Thoi, Phu Quoc Island, Kien Giang Province, Vietnam. The dried alga was extracted with methanol and partitioned between ether and water. The ether soluble fraction was subjected to silica gel column chromatography and further purified with preparative TLC to give these metabolites. The structures were established using basic ^{1}H, ^{13}C NMR, COSY, HSQC, and NOESY experiments.

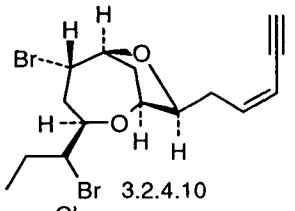


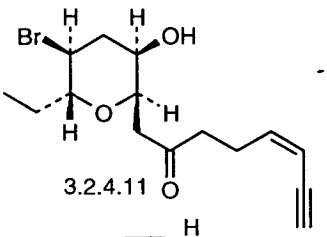
In 1996 Suzuki *et al.*[63] reported a new C_{15} nonterpenoid acetylenic compound, neoisoprelaurefucin (3.2.4.10) from *Laurencia nipponica*. Two samples were collected at Naoetsu and Kashiwazaki, Niigata Prefecture, Japan. The dried algae were extracted with methanol and partitioned between ether and water. The ether soluble portion was shaken with base. The neutral extract was subjected to silica gel column and further submitted to preparative TLC to give (3.2.4.10). Both samples showed almost identical TLC behaviour. COSY, HMBC, NOESY and NOE difference experiments established its structure.

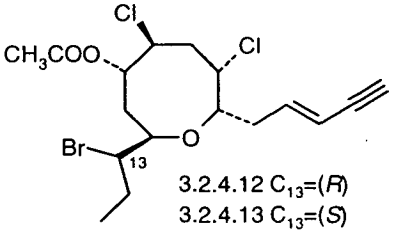
In 1997 Suzuki *et al.*[41] reported a new halogenated C_{15} acetogenin, scanlonenyne (3.2.4.11) from *Laurencia obtusa*, which was collected at Scanlon's Island, Ireland. Silica gel column and preparative TLC were used to purify the compound.

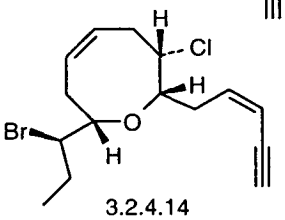
In 1997 two known acetylenes, 13-epilaurencienyne (3.2.4.12), and laurencienyne (3.2.4.13) were also reported from *Laurencia obtusa* by Imre *et al.*[45] The seaweed was collected from three different sites of Aegean coast, Turkey. The air-dried samples were extracted with chloroform-methanol (2:1) and chromatographed on silica gel column. Further purification with normal phase HPLC was carried out to give these metabolites. 13-Epilaurencienyne (3.2.4.12) showed two and a half times more activity than its stereoisomer, laurencienyne (3.2.4.13) towards brine shrimp *Artemia salina* having an LC_{50} of 111.9 compared to 280.1 mg/L.

In 1997 San-Martin *et al.*[64] reported a new C_{15} acetogenin, (3Z)-13-epipinnatifidenyne (3.2.4.14), pacifenol (3.2.1.45) as a major component, prepacifenol (3.2.1.44), deoxyprepacifenol (3.2.1.41), laurenisol and 9-hydroxy-4,10-dibromo-3-chloro-α-chamigrene (3.2.1.53) from *Laurencia claviformis*. The alga was collected at low tide pools at Vaihu, Easter Island, Chile. Silica gel column chromatography and recrystallization were used for a purification of 3.2.4.14.


3.2.4.10

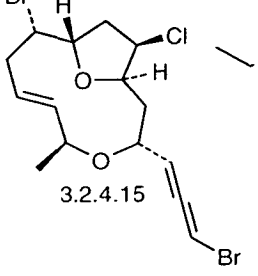

3.2.4.11

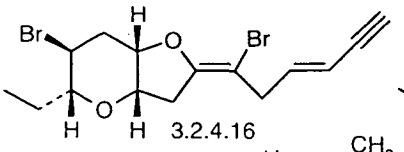

3.2.4.12 C_{13}=(*R*)
3.2.4.13 C_{13}=(*S*)

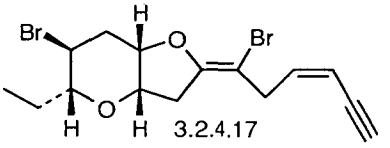

3.2.4.14

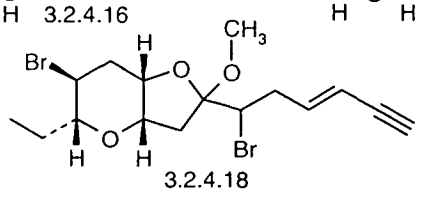
A new twelve-membered *O*-bridged cyclic ether, obtusallene IV (3.2.4.15) was reported from *Laurencia obtusa* by Guella *et al.*[65] The alga was collected from Kas in the Turkish Mediterranean.

In 1999 Takahashi *et al.*[66] reported three brominated C_{15} nonterpenoids, japonenyne A (3.2.4.16), japonenyne B (3.2.4.17), and japonenyne C (3.2.4.18), aplysiadiol (3.2.2.27), and 2,10-dibromo-3-chloro-α-chamigrene (3.2.1.36) from *Laurencia japonensis*, which was collected from several locations in Japan. HMBC, HSQC, and NOESY experiments were carried out to establish the structures.

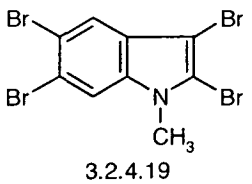

3.2.4.15

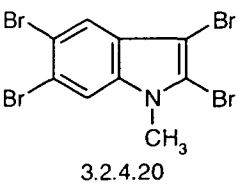

3.2.4.16


3.2.4.17


3.2.4.18

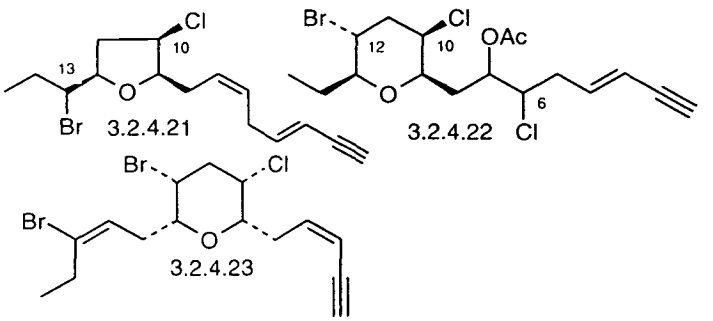
In 1999 Masuda *et al.*[67] reported two polybrominated indoles, 1-methyl-2,3,5,6-tetrabromoindole (3.2.4.19) and 2,3,5,6-tetrabromoindole (3.2.4.20) from *Laurencia similis*, which was collected from Borneo, Malaysia.


3.2.4.19


3.2.4.20

Two new C_{15} acetogenins, bisezakyne A (3.2.4.21) and bisezakyne B (3.2.4.22) and one known, dactylyne (3.2.4.23) were reported from *Laurencia* sp. by Suzuki *et al.*[68] The specimen was collected from Bisezaki, Motobu, Okinawa Prefecture, Japan. The dried alga

was extracted with methanol and partitioned with ether and water. The organic fraction was subjected to silica gel column chromatography and further submitted to preparative TLC to give (3.2.4.21-3.2.4.23). ^{1}H, ^{13}C NMR, COSY, HSQC, NOESY, HMBC spectroscopy were used to establish the structures. Moreover, use was made of ^{13}C NMR isotope shifts. The standard Lorentzian-Gaussian resolution enhancement procedure was used before Fourier transformation to achieve a better separation of $^{37}Cl/^{35}Cl$ and $^{81}Br/^{79}Br$ splittings. The signal of bisezakyne-A (3.2.4.21) at 60.4 ppm (C-10, an chlorine attached) showed an isotopic shift of 8.53 ppb (0.858 Hz) with a peak intensity ratio of 3.06:1 ($^{37}Cl:^{35}Cl$). While the signal of the same compound at 59.0 ppm (C-13, a bromine attached) showed as isotopic shift of 0.86 ppb (0.086 Hz) with a peak intensity ratio of 1:1.008 ($^{81}Br:^{79}Br$). On the other hand, the signal of bisezakyne-B (3.2.4.22) at 62.7 ppm (C-6, an chlorine attached) and at 61.2 ppm (C-10, an chlorine attached) displayed isotopic shifts of 7.90 ppb (0.793 Hz) with a peak intensity ratio of 2.55:1 and 7.74 ppb (0.779 Hz) with a peak intensity ratio of 2.75:1, respectively. The isotope shift of the signal at 46.3 ppm (C-12, a bromine attached) was not detected. Therefore, the ^{13}C NMR isotope shifts indicated that which halogen (e.g. Cl or Br) was attached to which carbon by splitting pattern and the peak intensity ratio.



3.3 Results and discussion

Laurencia filiformis and *Aplysia parvula* were collected at Taroona Beach, Hobart, Tasmania by scuba diving. However, when the sea hares *Aplysia parvula* were collected, there was not much *Laurencia filiformis* around. Whole freeze-dried *Aplysia parvula* was extracted with petroleum ether, dichloromethane, and methanol. The petroleum ether extract was purified by open-column flash silica gel chromatography. One fraction was recrystallized to give a new major metabolite, 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1), 0.073% yield (base on the sea hare dry weight) as white needles. Further purification of the remainder of the petroleum extract fractions by medium pressure

liquid chromatography and preparative TLC gave 2,10-dibromo-3-chloro-7-chamigrene (3.3.2), 0.010% yield (base on the sea hare dry weight), deoxyprepacifenol (3.2.1.41), 0.042% yield (base on the sea hare dry weight), pacifenol (3.2.1.45), and 3-bromo-4-(dibromomethylene)-2-pentylcyclopent-2-en-1-one (3.3.3). The dichloromethane extract was further purified through silica gel and then Sephadex LH-20 column chromatography. The purple pigment, aplysioviolin (3.3.4), 0.014% yield (base on the sea hare dry weight) was obtained.

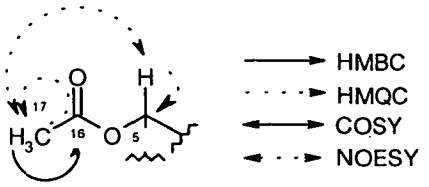
Freeze-dried *Laurencia filiformis* was extracted with dichloromethane. Purification by open-column flash silica gel and then medium pressure liquid chromatography yielded the sesquiterpenes (3.3.1), 0.230% yield (base on the alga dry weight), (3.3.2), 0.036% yield (base on the alga dry weight), (3.2.1.41), 0.135% yield (base on the alga dry weight), (3.2.1.45), 0.143% yield (base on the alga dry weight), and pentadecanal (3.3.10), 0.005% yield (base on the alga dry weight) and its aldol product, (*E*)-2-tridecyl-2-heptadec-2-enal (3.3.11), 0.010% yield (base on the alga dry weight). Their structures were elucidated by interpretation of their spectral data.

3.3.1 The structure of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1)

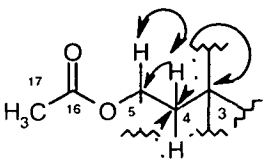
High Resolution LSIMS gave $C_{17}H_{24}Br_2ClO_3$ $[M+H]^+$ which corresponds to a molecular formula of $C_{17}H_{23}Br_2ClO_3$, indicating five degrees of unsaturation. The EI mass spectrum showed a molecular ion cluster of Br_2Cl halogen species with the value of 467.95 (0.092), 470.05 (0.175), 471.95 (0.132), 474.15 (0.049).

The ^{13}C NMR spectrum (Figure 3.3.1.1, CD_3Cl as solvent, Table 3.3.1.1) showed seventeen carbons corresponding with the formula established by mass spectrometry. The DEPT spectrum displayed five methyls, two methylenes, four methines, and six quaternary carbons.

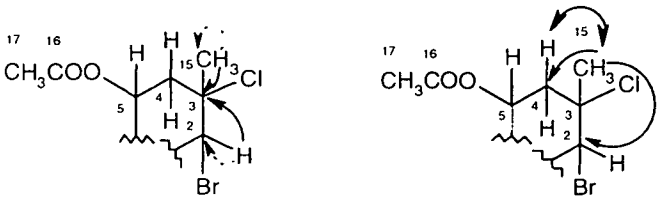
An acetate group was evident by a fragment ion of m/z 43 in the mass spectrum and an infrared absorption at ν_{max} 1744 cm^{-1}. The acetate carbonyl carbon (C-16) at 168.9 ppm showed a connection by HMBC to the methyl protons (H-17)$_3$ at 2.03 ppm. The methine chemical shift of C-5, at 72.2 ppm and H-5, at 5.20 ppm suggested that it had an attached acetoxy group.[69] One bond connection of C-17 at 21.8 ppm to (H-17)$_3$ and C-5 to H-5 were detected by HMQC. NOESY data also confirmed that the acetoxy group was attached to C-5 since a cross peak occurred between H-5 to H-17.



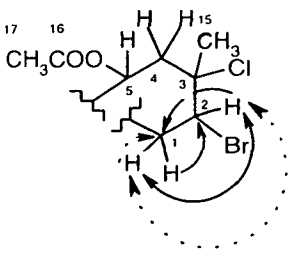
The methine carbon (C-5) was connected to the methylene protons $(H\text{-}4)_2$ at 2.55 ppm and these protons $(H\text{-}4)_2$ were also linked to a quaternary carbon (C-3) at 70.1 ppm by HMBC. COSY correlation between H-4 and H-5 was shown. In addition, the methylene carbon (C-4) at 43.6 ppm was connected to $(H\text{-}4)_2$ at 2.55 ppm by HMQC.



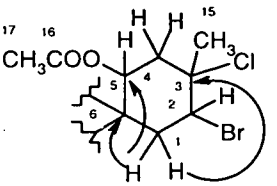
A connection of C-3 to a methine proton (H-2) at 4.70 ppm was established by HMBC. The chemical shift of C-2 at 62.0 and C-3 at 70.1 ppm indicated a bromo and a chloro substituent, respectively.[39] Moreover, HMBC displayed a linkage of the methyl protons $(H\text{-}15)_3$ at 1.76 ppm to C-3. These methyl protons $(H\text{-}15)_3$ showed a connection by HMQC to carbon (C-15) at 22.5 ppm. Similarly, the methine proton (H-2) at 4.70 ppm linkage to carbon (C-2) at 62.0 ppm was revealed by HMQC. Furthermore, connectivities between the methyl protons $(H\text{-}15)_3$ to C-4, as well as to C-2 were revealed by HMBC.

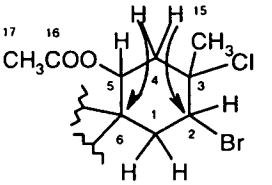


Next, HMBC exhibited a connection between H-2 to a methylene carbon (C-1) at 35.8 ppm and between the methylene protons $(H\text{-}1)_2$ at 2.45 ppm to carbon (C-2). HMQC established a one bond connection of C-1 at 35.8 ppm to the methylene protons $(H\text{-}1)_2$ at 2.45 ppm. In addition, H-1 and H-2 were connected by COSY and NOESY experiments.

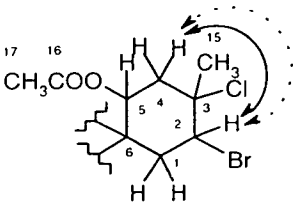


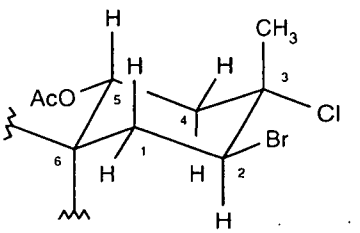
The methylene protons $(H\text{-}1)_2$ displayed connectivities by HMBC further to a quaternary carbon (C-6) at 51.2 ppm, to the methine carbon (C-5) and to the quaternary carbon (C-3). In addition, the quaternary carbon (C-6) established a link by HMBC to the methylene protons $(H\text{-}4)_2$ which also had a connection to the methine carbon (C-2).





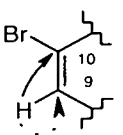
Then NOESY correlation occurred between H-2 and one of H-4 showing that both of these were axial. This relationship was confirmed by COSY. At this stage it was concluded that C-1 to C-6 formed the framework of a cyclohexane ring. These axial protons (H-4, H-2) implied a relative stereochemistry of fragment A as a chair conformation.





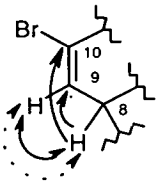
fragment A

An olefinic quaternary carbon (C-10) at 142.4 ppm had an attached bromine atom because of its low field chemical shift.[42, 49] The only olefinic methine carbon (C-9) at 124.4 ppm had its attached proton (H-9) at 6.25 ppm; this association was confirmed by HMQC. A connection by HMBC of H-9 to C-10 was established.

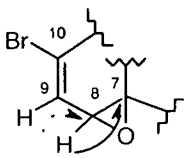


The connectivity of H-8 and H-9 was confirmed by both COSY and NOESY signals. Moreover, HMBC displayed a connection of H-8 to C-9 and H-8 to C-10. The H-9 proton signal was a doublet due to coupling to the methine proton (H-8), also doublet, at 3.04 ppm. The coupling constant for both doublets was 2.4 Hz, which indicated a dihedral angle of 55° by the vicinal Karplus correlation curve.[70, 71] Similarly, the dihedral angle was calculated as 64.5° by the simple Karplus equation.[72] Two reports[73, 74] recently refined the

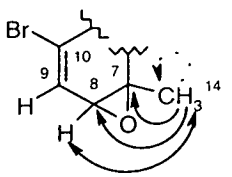
Karplus rule, but none of the model compounds contained a cyclohexene with an epoxide so the dihedral angle remains uncertain.

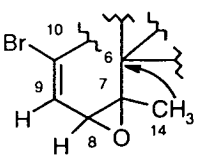


The bonding of the methine proton (H-8) at 3.04 ppm to carbon (C-8) at 57.9 ppm was shown by HMQC. The downfield chemical shifts of the methine carbon (C-8) and the quaternary carbon (C-7) at 57.9 and 59.6 ppm, respectively, indicated an epoxy group connection.[49] Moreover, HMBC displayed a connection of H-8 to C-7.

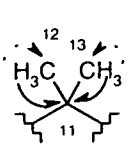


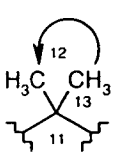
Next HMBC showed a connection of C-7 to $(H\text{-}14)_3$ at 1.63 ppm and C-8 to $(H\text{-}14)_3$. While a one bond connection of C-14 at 27.2 ppm to $(H\text{-}14)_3$ was shown by HMQC. Therefore, C-14 is a methyl that is joined to C-7. Long-range coupling of H-8 and $(H\text{-}14)_3$ was also displayed in the COSY spectrum. This established a connectivity of H-8 and $(H\text{-}14)_3$. In addition, the methyl protons $(H\text{-}14)_3$ had a long-range HMBC connectivity to the quaternary carbon (C-6).

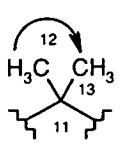




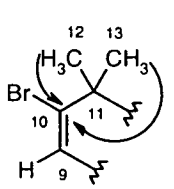
Two of the other methyl groups had a geminal relationship. The connections between C-12 at 28.5 ppm and $(H\text{-}12)_3$ at 1.29 ppm and between C-13 at 26.0 ppm and $(H\text{-}13)_3$ at 1.34 ppm were established by HMQC. The fact that both methyls were attached to a quaternary carbon (C-11) at 46.8 ppm was established by HMBC. Connectivities by HMBC between the methyl protons $(H\text{-}12)_3$ to C-13 and similarly between the protons $(H\text{-}13)_3$ to C-12 were revealed.

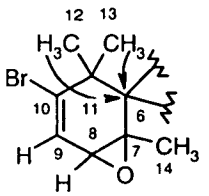




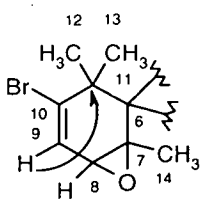


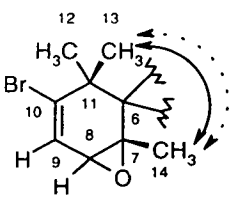
These methyl protons, $(H\text{-}12)_3$ and $(H\text{-}13)_3$ exhibited long-range HMBC connectivities to C-10. Long-range HMBC connectivities between the protons $(H\text{-}12)_3$ to C-6 and the protons $(H\text{-}13)_3$ to C-6 were also observed.





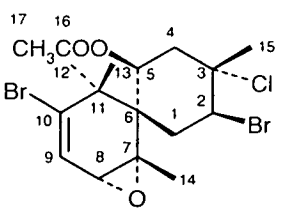
HMBC also showed a linkage between C-11 and H-9 thereby providing further evidence that C-11 and C-10 were joined. In addition, long-range COSY and NOESY signals of $(H\text{-}14)_3$ to $(H\text{-}13)_3$ were also detected. The latter implied a relative stereochemistry of $(H\text{-}14)_3$ and $(H\text{-}13)_3$ as shown. At this point it was concluded that C-6 to C-11 formed the framework of a cyclohexene ring (fragment B).





fragment B

Since C-6 was common to both rings, the compound had a spiro arrangement. The protons $(H\text{-}1)_2$ also linked to C-7 and C-14 by long-range HMBC connectivities. Therefore, fragment A and fragment B were joined and formed a chamigrene structure. A single crystal X-ray result confirmed these features and established the absolute configuration.



3.3.1

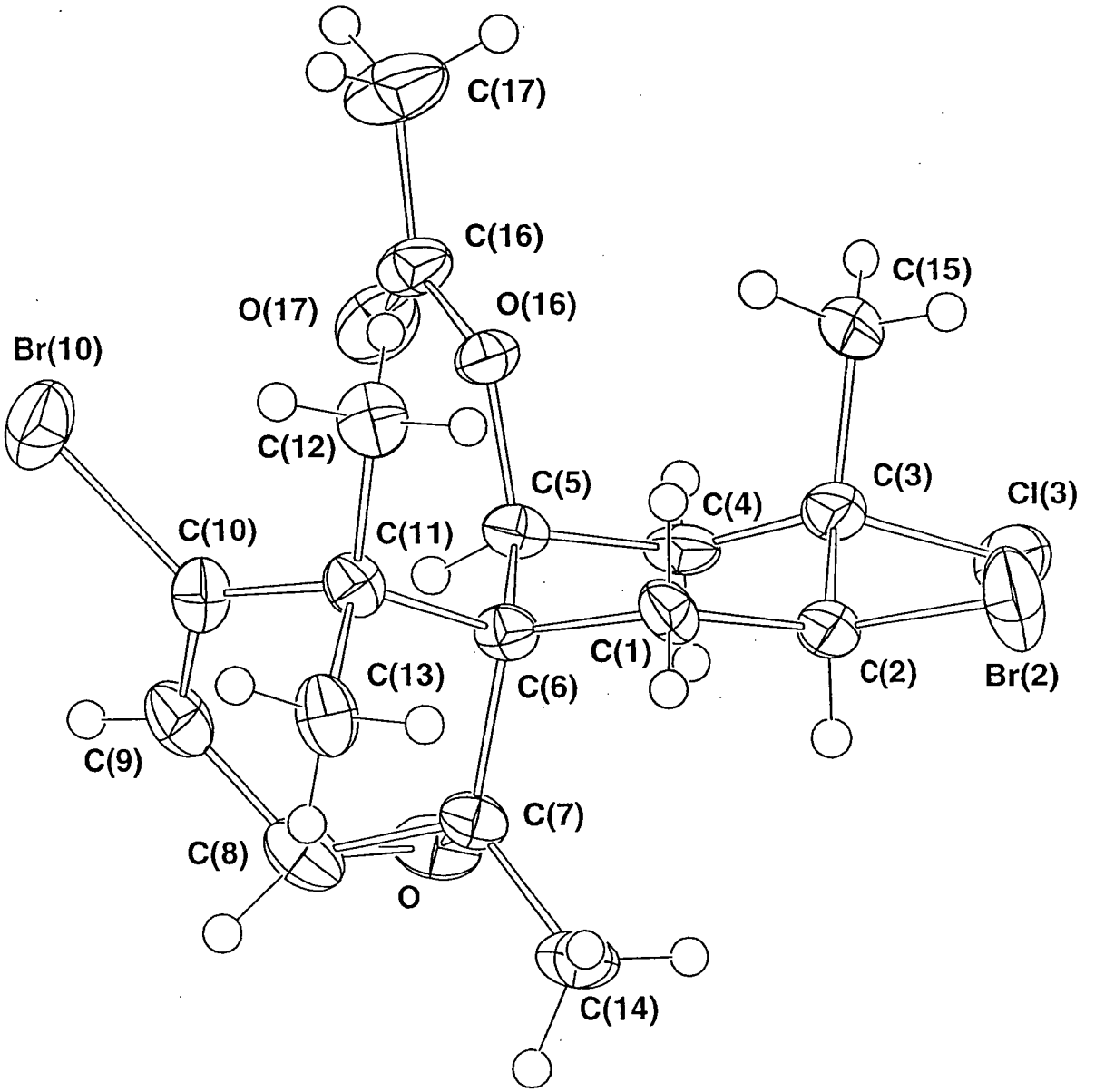


Figure 3.3.1.1 X-ray result of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene. Projection and parameters of the molecule showing 50% probability amplitude displacement parameters for the non-hydrogen atoms, hydrogen atoms having arbitrary radii of 0.1 Å.

Table 3.3.1.1. ^{1}H and ^{13}C NMR data of (3.3.1) and (3.3.2) in $CDCl_3$.

No.	(3.3.1)			(3.3.2)		
	^{1}H, *J* (Hz)	^{13}C, DEPT	HMBC	^{1}H, *J* (Hz)	^{13}C, DEPT	HMBC
1	2.45 m	35.8, CH_2	2	2.60 m	36.2, CH_2	-
2	4.70 dd, 5.8, 11.6	62.0, CH	1, 4, 15	4.90 dd, 7.6, 3.2	63.0, CH	-
3	-	70.1, C	1, 2, 4, 15	-	71.1, C	2, 4
4	2.55 m	43.6, CH_2	15	2.20 m	39.4, CH_2	-
5	5.20 dt, 1.6, 3.2	72.2, CH	1, 4	1.60 m	31.6, CH_2	4
6	-	51.2, C	1, 4, 12, 13, 14	-	47.6, C	4, 13, 14
7	-	59.6, C	1, 8, 9, 14	-	139.5, C	9, 14
8	3.04 d, 2.4	57.9, CH	14	5.21 br, dt	122.7, CH	14
9	6.25 d, 2.4	124.4, CH	8	2.30 m	40.3, CH_2	12
10	-	142.4, C	8, 9, 12, 13	4.50 dd, 6.8, 4.0	60.8, CH	12, 13
11	-	46.8, C	9, 12, 13	-	42.8, C	13
12	1.29 s	28.5, CH_3	13	1.69 s	24.1, CH_3	13
13	1.34 s	26.0, CH_3	12	0.92 s	17.1, CH_3	10
14	1.63 s	27.2, CH_3	1, 2, 4	1.98 s	26.0, CH_3	-
15	1.76 s	22.5, CH_3	-	1.20 s	24.6, CH_3	2
16	-	168.9, C	17			
17	2.03 s	21.8, CH_3	-			

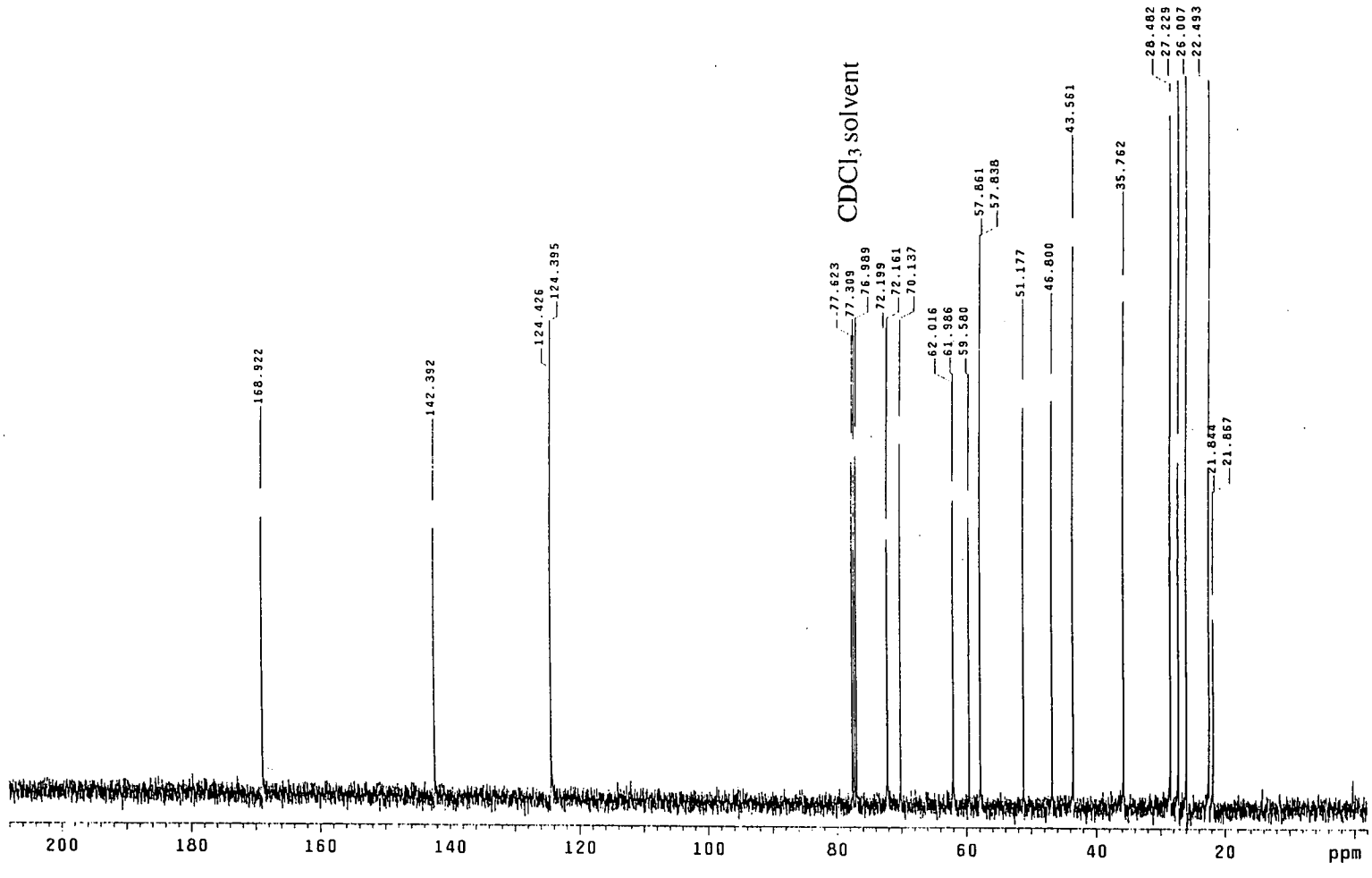


Figure 3.3.1.2 ^{13}C NMR spectrum of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

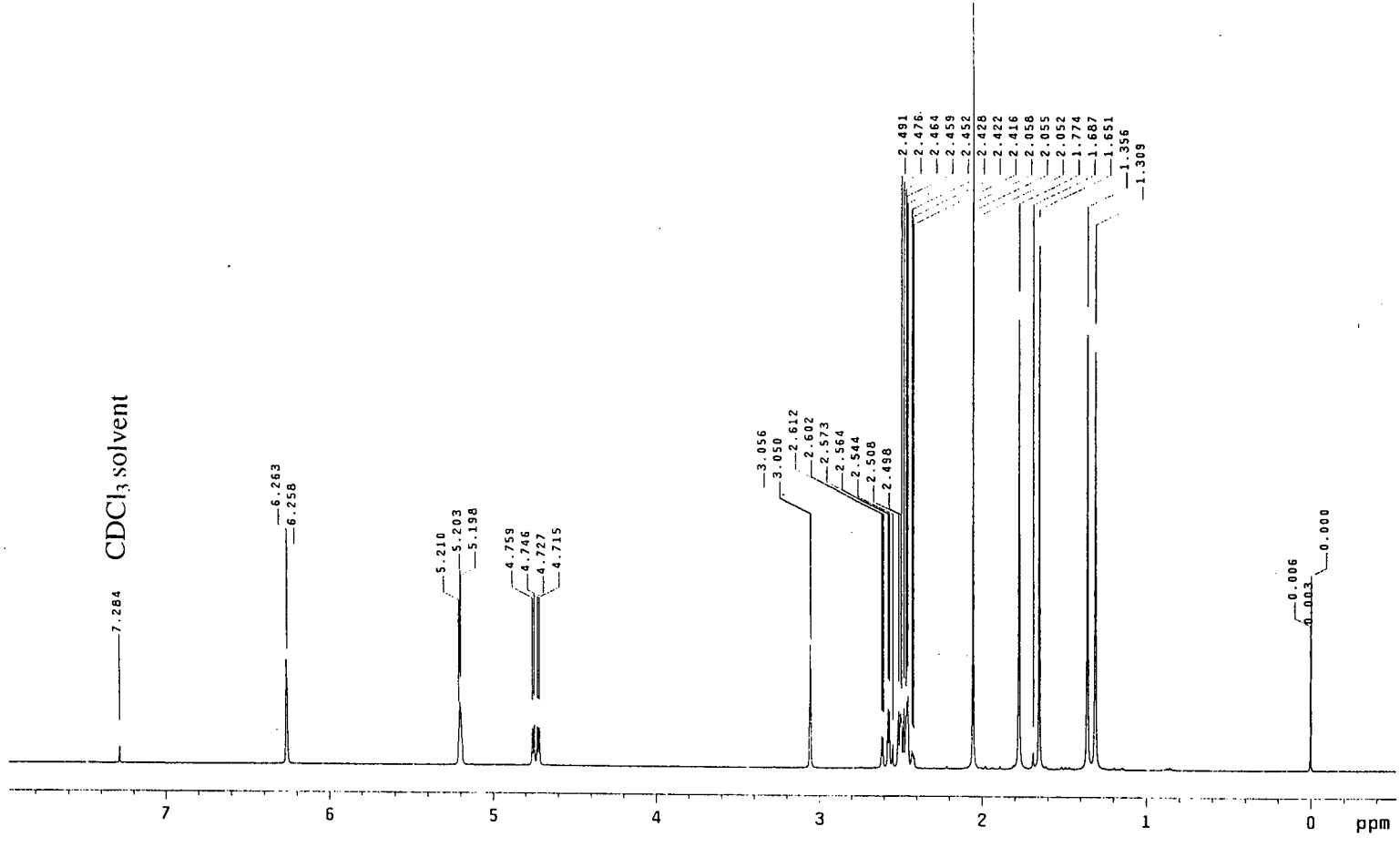


Figure 3.3.1.3 ^{1}H NMR spectrum of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

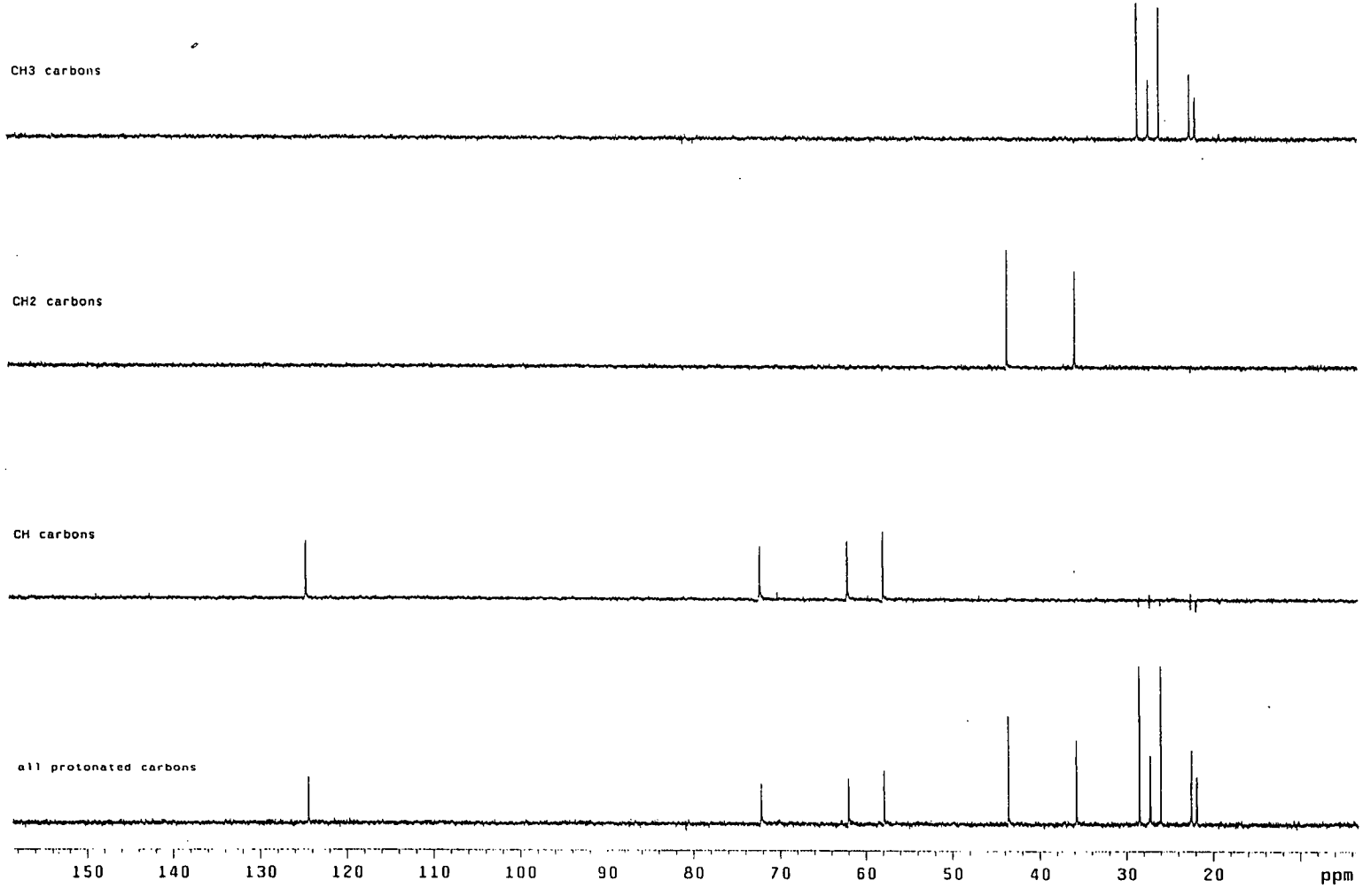


Figure 3.3.1.4 DEPT experiment of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

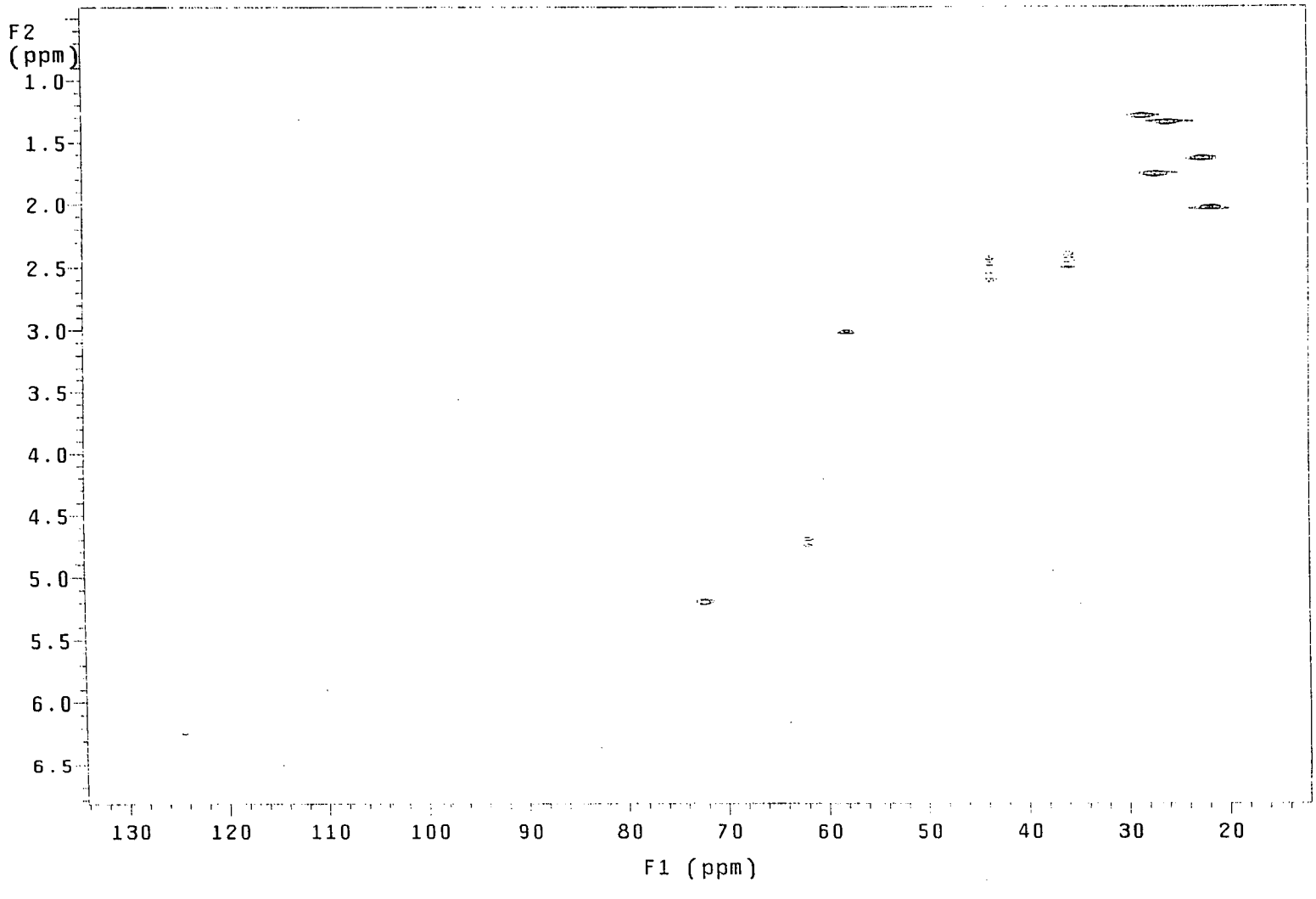


Figure 3.3.1.5 HMQC experiment of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

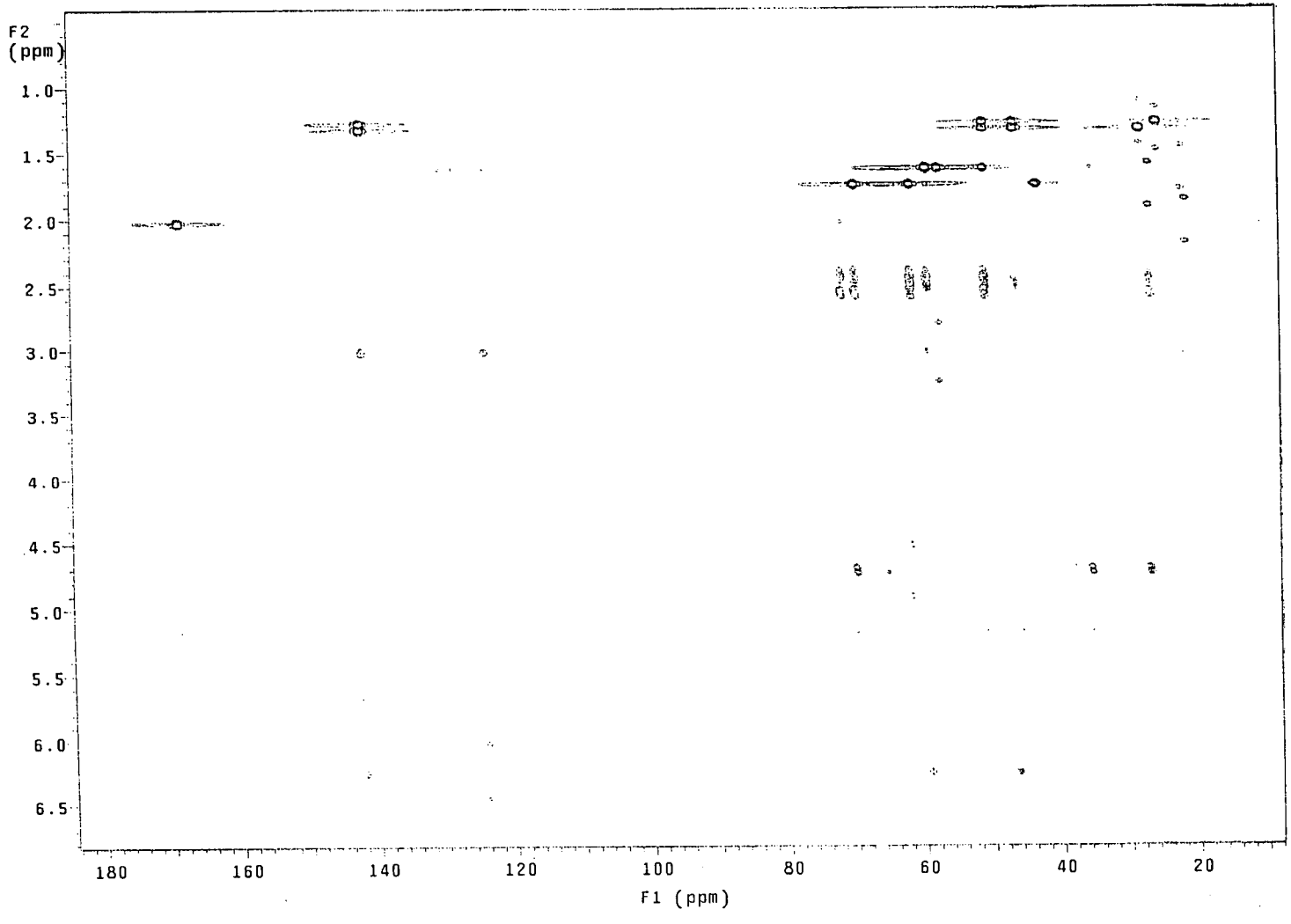


Figure 3.3.1.6 HMBC experiment of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

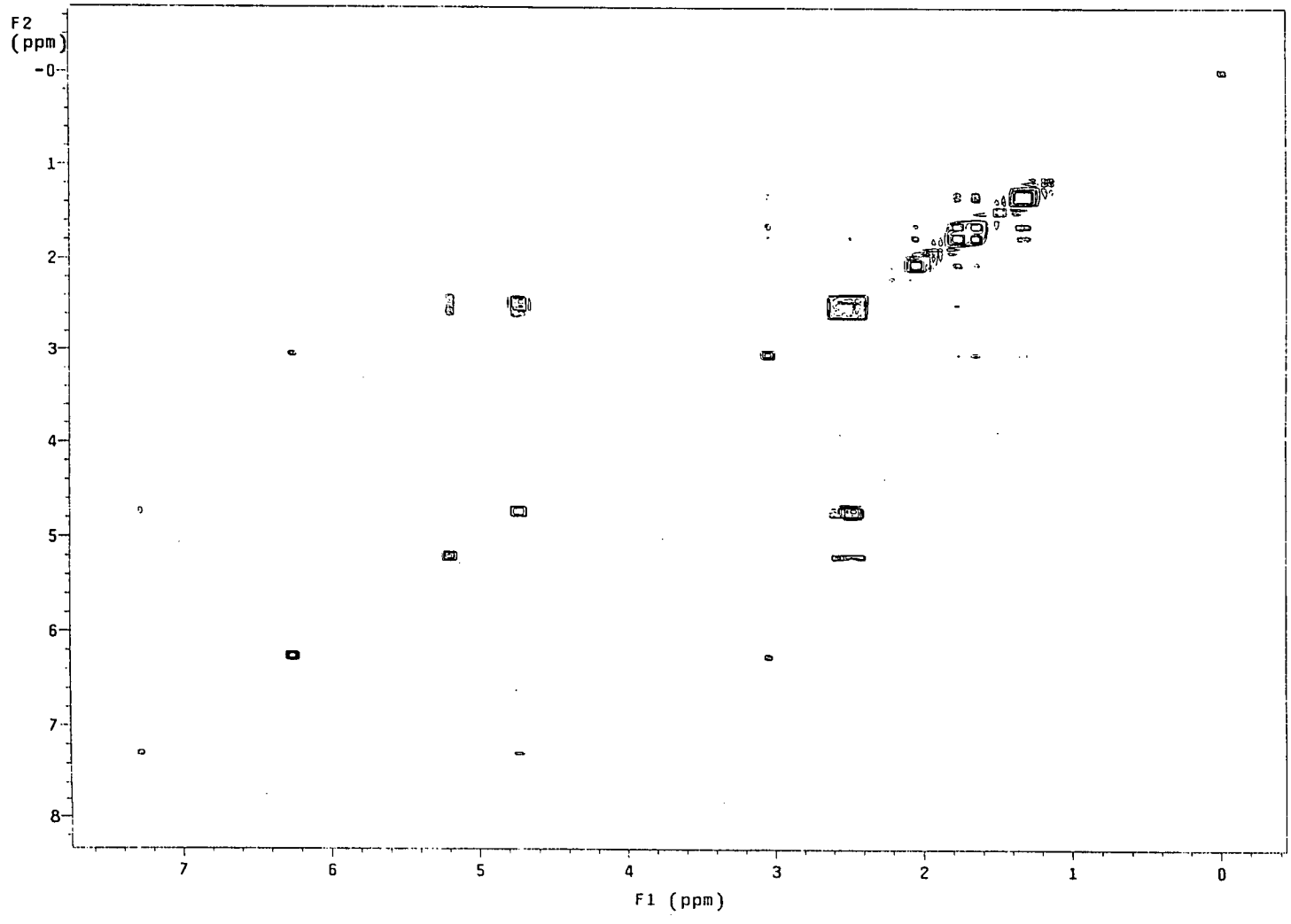


Figure 3.3.1.7 COSY experiment of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

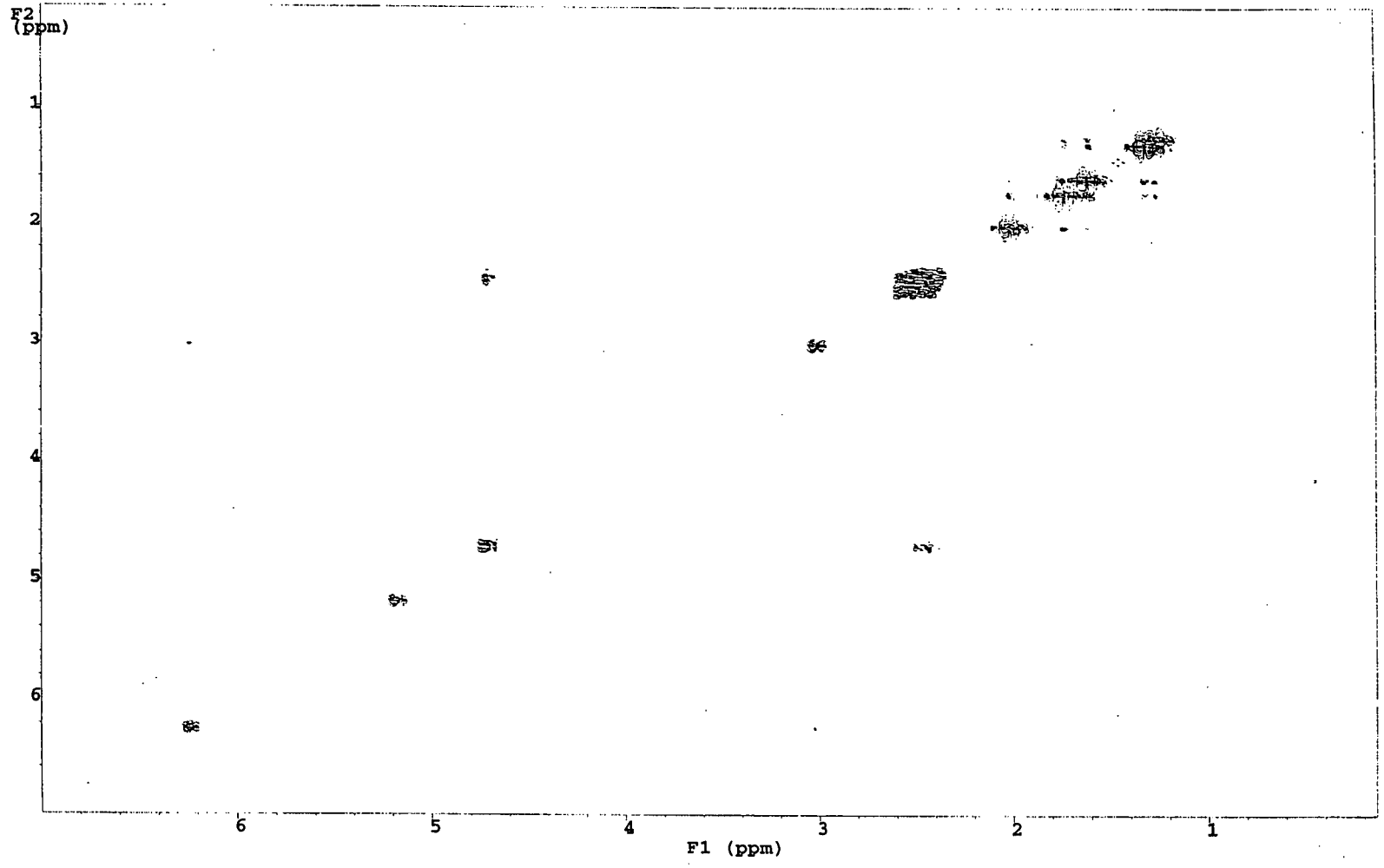
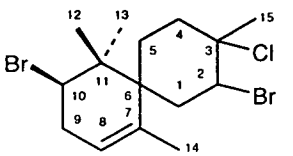


Figure 3.3.1.8 NOESY experiment of 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1).

3.3.2 The structure of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2)

The sesquiterpene (3.3.2) had been previously found in the red seaweed *Laurencia* sp. by Howard and Fenical.[75] The chamigrene (3.3.2) in this study was separated from both the sea hare *A. parvula* and the red seaweed *L. filiformis* as 0.010 and 0.036% yield (base on the sea hare and the seaweed dry weight, respectively). ^{1}H, ^{13}C NMR as well as HMQC and HMBC experiments were used to establish its structure in this study.

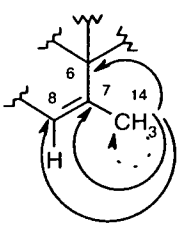


3.3.2

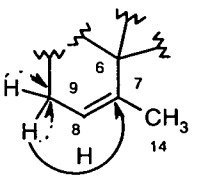
The EI mass spectrum showed a molecular ion cluster of Br_2Cl halogen species with the value of 396 (0.32): 398 (0.75): 400 (0.44): 402 (0.13). A molecular formula of $C_{15}H_{23}Br_2Cl$ by peak matching indicated three degrees of unsaturation.

The ^{13}C NMR spectrum (Figure 3.3.2.1, $CDCl_3$ as solvent, Table 3.3.1.1) showed fifteen carbons corresponding to the formula. The DEPT spectrum displayed four methyls, four methylenes, three methines, and four quaternary carbons.

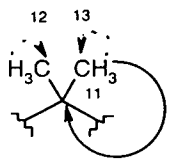
The only olefinic quaternary carbon (C-7) at 139.5 ppm was connected to the methyl protons $(H\text{-}14)_3$ at 1.98 ppm by HMBC. These protons $(H\text{-}14)_3$ had a one bond connection to carbon (C-14) at 26.0 ppm by HMQC. A methine olefinic carbon (C-8) at 122.7 ppm was linked by HMBC to the protons $(H\text{-}14)_3$ and these protons $(H\text{-}14)_3$ were also connected to a quaternary carbon (C-6) at 47.6 ppm by HMBC. HMQC confirmed a linkage between C-8 at 122.7 ppm and H-8 at 5.21 ppm.



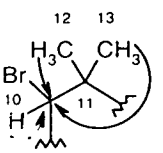
A connection by HMBC between the methylene protons $(H\text{-}9)_2$ at 2.30 ppm and carbon (C-7) was shown. HMQC exhibited a link of C-9 at 40.3 ppm to the methylene protons $(H\text{-}9)_2$ at 2.30 ppm.



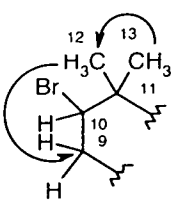
Two methyl groups had a geminal relationship. HMQC established connectivities of C-12 at 24.1 ppm to $(H\text{-}12)_3$ at 1.69 ppm and C-13 at 17.1 ppm to $(H\text{-}13)_3$ at 0.92 ppm. The methyl protons $(H\text{-}13)_3$ were connected to a quaternary carbon (C-11) at 42.8 ppm by HMBC.

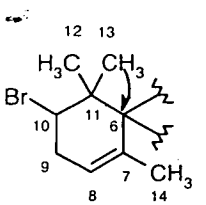


HMBC displayed connectivities between these protons ($(H\text{-}12)_3$, $(H\text{-}13)_3$) and a methine carbon (C-10) at 60.8 ppm. A methine proton (H-10) at 4.50 ppm was connected to C-10 at 60.8 ppm by HMQC. Since both the carbon (C-10) and hydrogen (H-10) of this methine had low field chemical shifts, thus it had an attached bromine atom.

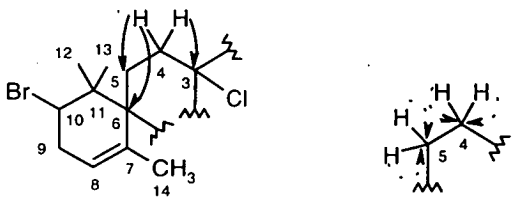


Moreover, the protons $(H\text{-}13)_3$ were linked to carbon (C-12) by HMBC. The methyl protons $(H\text{-}12)_3$ was connected to the methylene carbon (C-9) by long-range HMBC. Next, the quaternary carbon (C-6) was connected to the protons $(H\text{-}13)_3$ by HMBC. Therefore, C-6 to C-11 formed a cyclohexene ring.

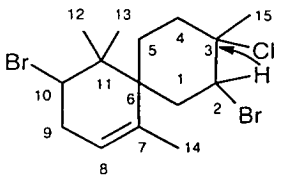




The quaternary carbon (C-6) displayed a further connection by a long-range HMBC to the methylene protons $(H\text{-}4)_2$ at 2.20 ppm. Then these methylene protons $(H\text{-}4)_2$ were linked to a methylene carbon (C-5) at 31.6 ppm and to a quaternary carbon (C-3) at 71.1 ppm. The quaternary carbon (C-3) had an attached chlorine atom because of its downfield chemical shift. HMQC showed one bond connections between C-4 at 39.4 ppm to $(H\text{-}4)_2$ at 2.20 ppm and C-5 at 31.6 ppm to $(H\text{-}5)_2$ at 1.60 ppm.



A methine proton (H-2) at 4.90 ppm was connected to the quaternary carbon (C-3) by HMBC. Unfortunately, it had no further observed HMBC connections. However, only a methylene carbon (C-1) and a methyl carbon (C-15) were left over. It is more likely that the methyl carbon (C-15) was connected to the quaternary carbon (C-3) and the methylene carbon (C-1) was linked between C-6 and C-2 in order to form a cyclohexane ring (C-1 to C-6). Therefore, the compound (3.3.2) had a spiro arrangement similar to the compound (3.3.1). ^{1}H NMR data of the compound (3.3.2) was identical to the literature.[62]



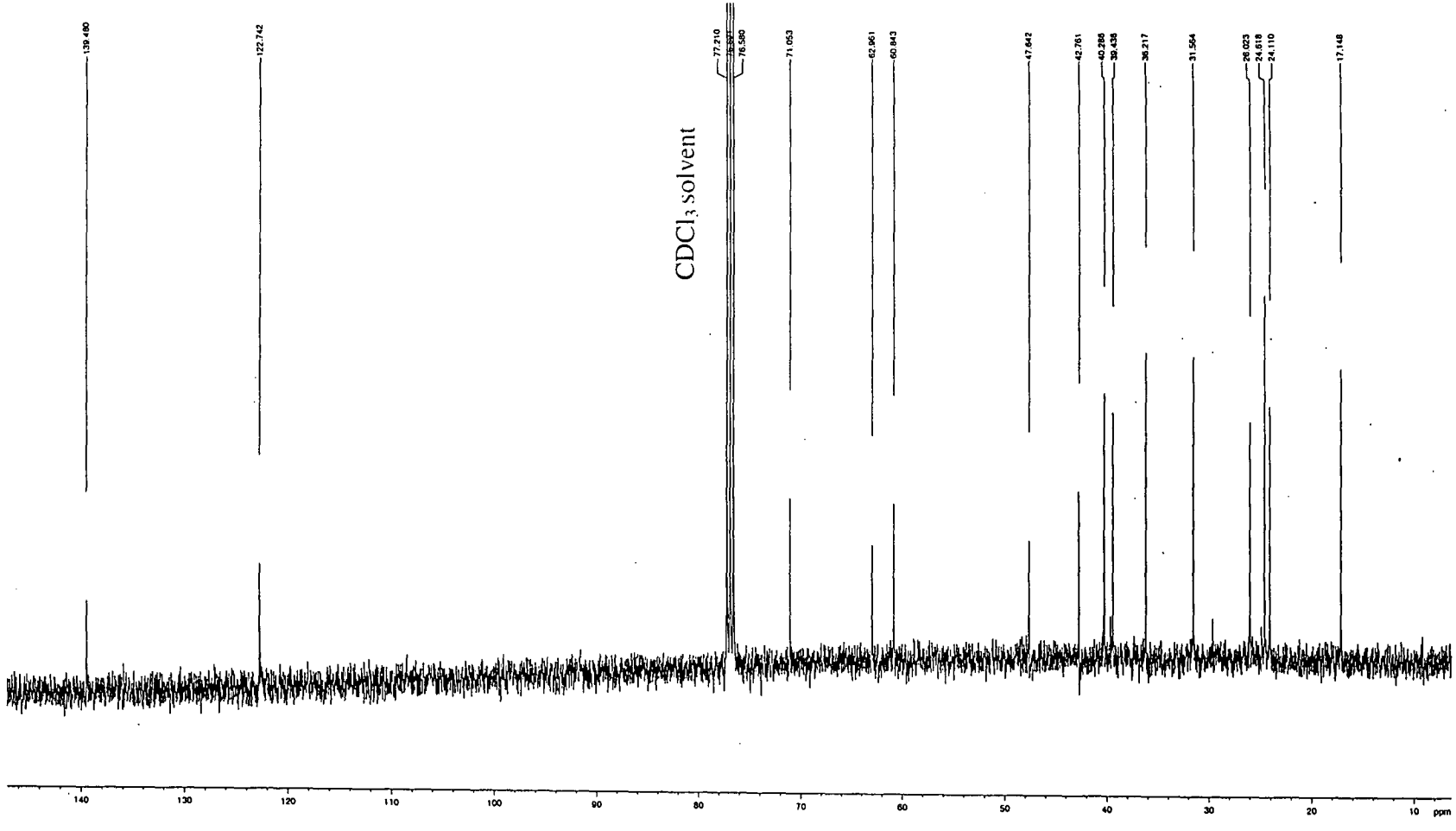


Figure 3.3.2.1 ^{13}C NMR spectrum of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2).

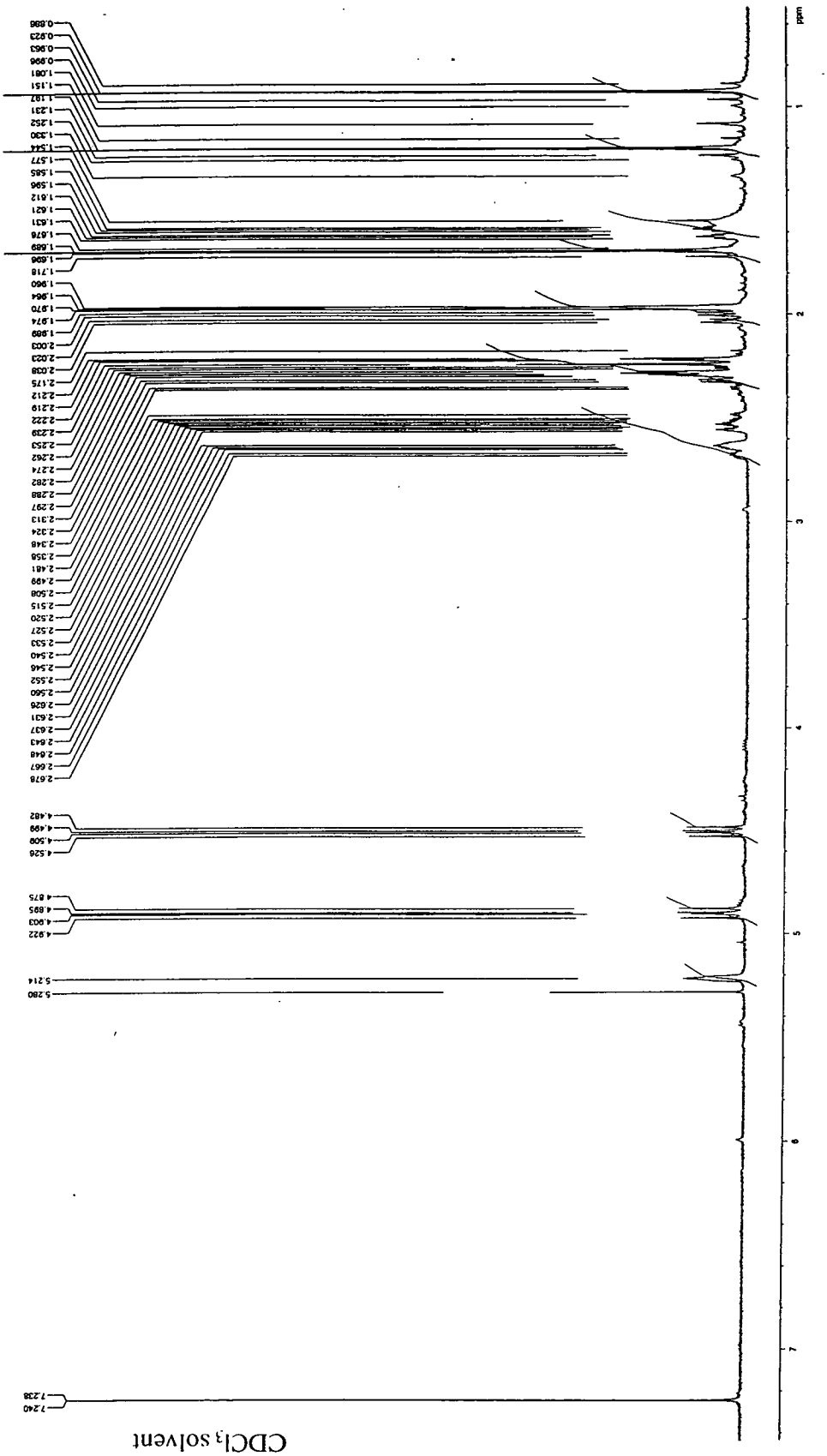


Figure 3.3.2.2 ^{1}H NMR spectrum of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2).

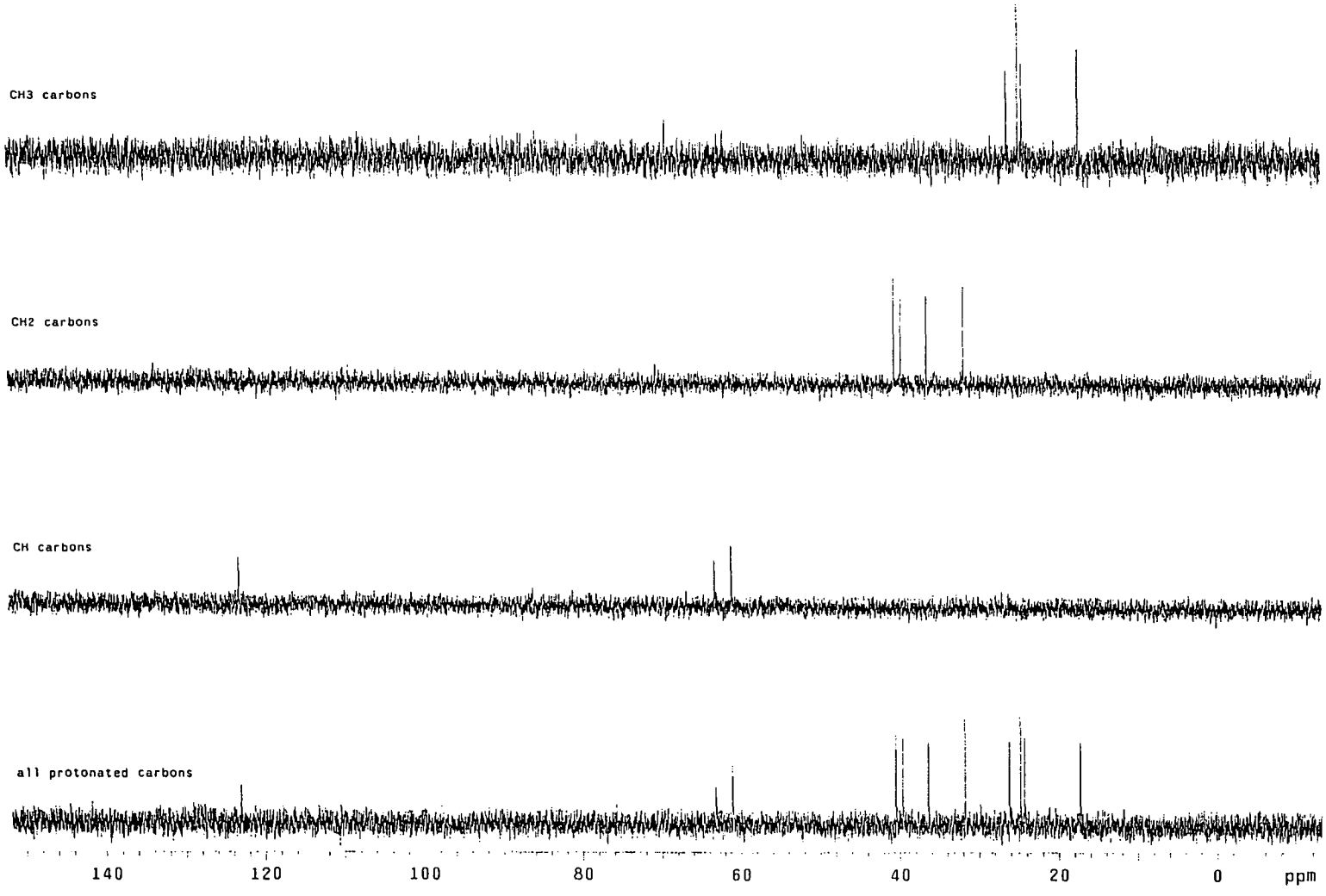


Figure 3.3.2.3 DEPT experiment of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2).

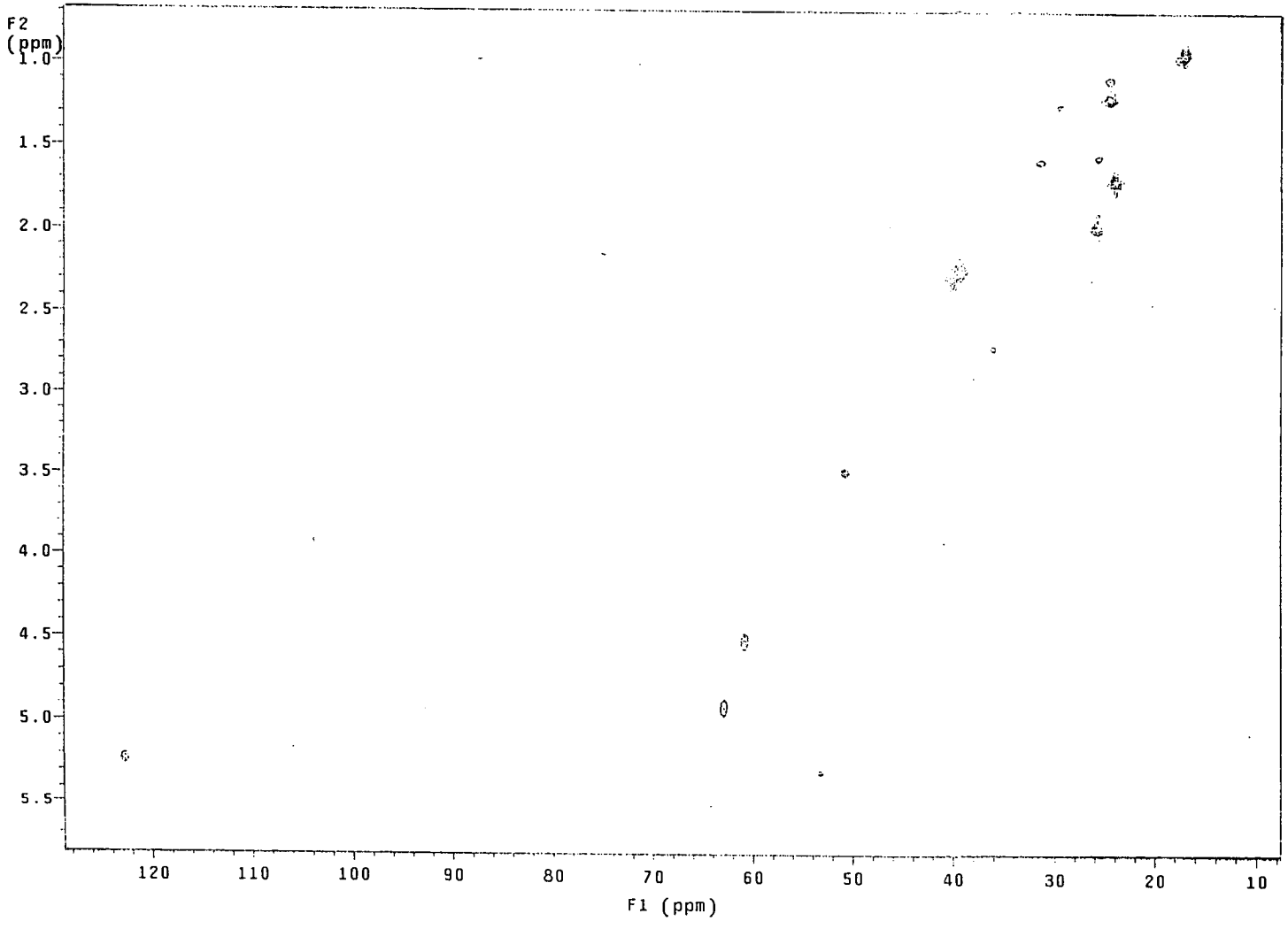


Figure 3.3.2.4 HMQC experiment of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2).

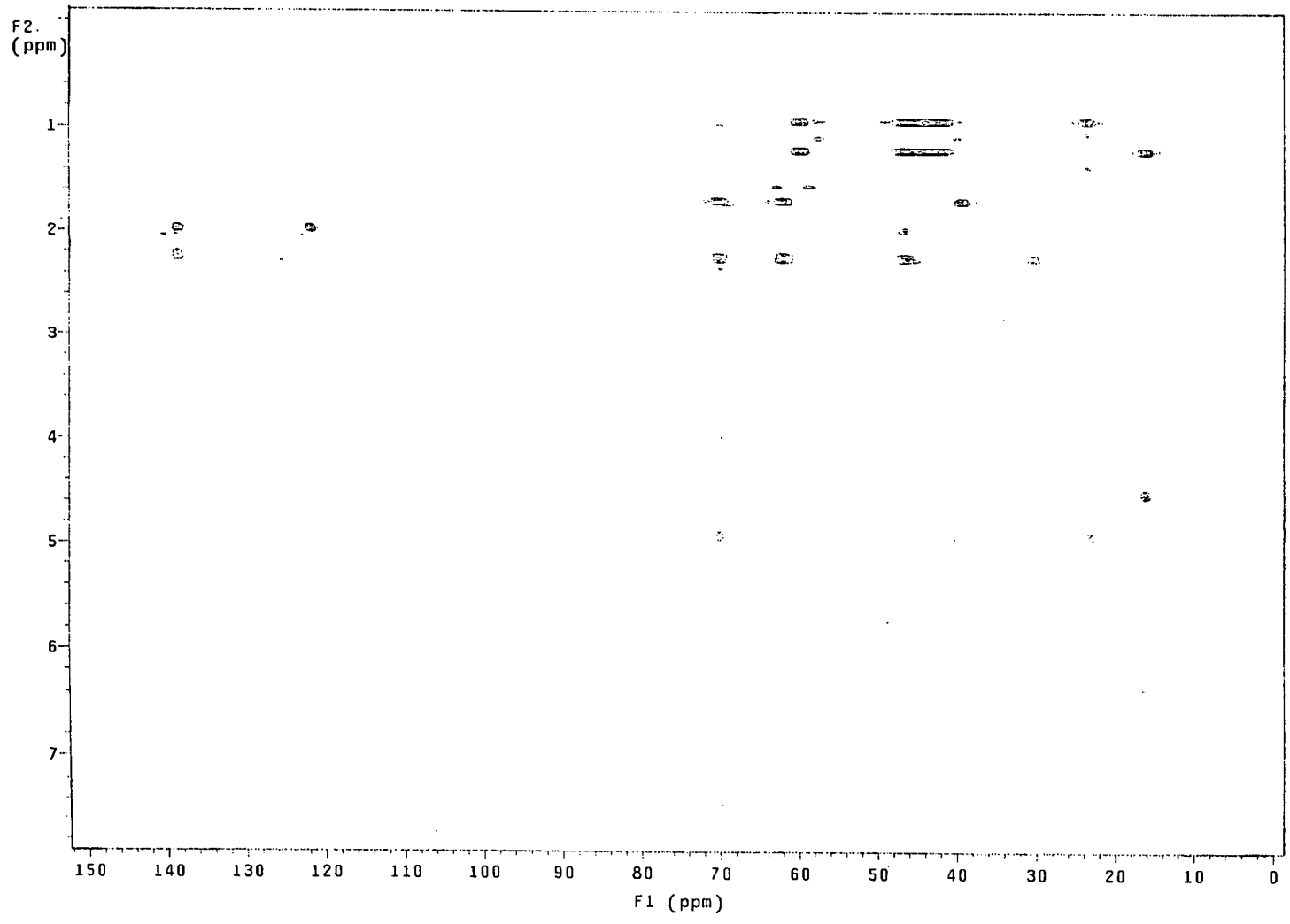
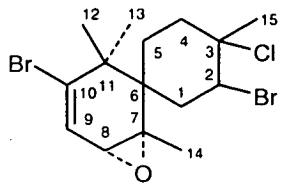


Figure 3.3.2.5 HMBC experiment of 2,10-dibromo-3-chloro-7-chamigrene (3.3.2).

3.3.3 The structure of deoxyprepacifenol (3.2.1.41).

Deoxyprepacifenol (3.2.1.41) had been previously isolated from the red seaweed *Laurencia* species.[76] In this study, deoxyprepacifenol was isolated from both the sea hare *A. parvula* in 0.042% yield (base on the sea hare dry weight) and *L. filiformis* in 0.135% yield (base on the red seaweed dry weight).



3.2.1.41

The EI mass spectrum showed a molecular ion cluster of Br_2Cl halogen species with the value of 410 (1.72), 412 (4.11), 414 (3.01), 416 (0.68). A molecular formula of $C_{15}H_{21}Br_2ClO$ by peak matching indicated four degrees of unsaturation.

The ^{13}C NMR spectrum showed fifteen carbons corresponding with the formula and the literature values as shown in Table 3.3.3.1. The DEPT spectrum displayed four methyls, three methylenes, three methines, and five quaternary carbons. The ^{1}H NMR spectrum also corresponded well with the literature values as shown in Table 3.3.3.1.

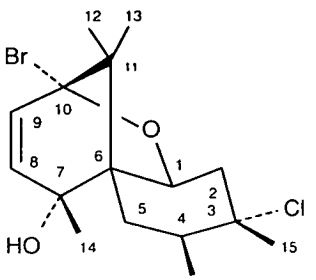
Table 3.3.3.1. ^{1}H and ^{13}C NMR data of (3.2.1.41) in $CDCl_3$ and literature values.

No.	3.2.1.41		Deoxyprepacifenol literature value[78]	
	^{1}H, *J* (Hz)	^{13}C, DEPT	^{1}H, *J* (Hz)	^{13}C
1	2.10-2.42 m	39.7, CH_2	2.08-2.46 m	39.62
2	4.68 dd, 4.6, 13.0	63.3, CH	4.68 dd, 6, 12	63.06
3	-	71.4, C	-	71.08
4	2.10-2.42 m	39.1, CH_2	2.08-2.46 m	39.08
5	2.10-2.42 m	26.0, CH_2	2.08-2.46 m	25.85
6	-	49.3, C	-	49.01
7	-	58.4, C	-	58.10
8	2.92 d, 2.8	56.5, CH	2.94 d, 4	58.35
9	6.22 d, 2.8	124.3, CH	6.24 d, 4	124.18
10	-	143.9, C	-	143.70
11	-	46.0, C	-	46.10
12	1.18* s	24.1*, CH_3	1.18-1.20 s	23.86
13	1.34* s	26.0*, CH_3	1.18-1.20 s	23.46
14	1.61 s	24.5, CH_3	1.63 s	24.33
15	1.67 s	24.9, CH_3	2.08-2.46 m	24.79

*These chemical shifts may be interchanged.

3.3.4 The structure of pacifenol (3.2.1.45).

The known sesquiterpene pacifenol (3.2.1.45) was found in both the sea hare *A. parvula* and the red seaweed *L. filiformis*. Its presence in the sea hare was determined by GCMS of a silica gel fraction of the crude extract while it was isolated in 0.143% yield (base on the seaweed dry weight) from *L. filiformis*. Pacifenol (3.2.1.45) was previously isolated from a red seaweed *Laurencia* species.[77,78]



3.2.1.45

The EI mass spectrum showed the value of m/z 408 (M^+-H_2O, 1.3), 410 (2.4), 412 (1.7), 372 (1.5), 374 (1.9), 376 (0.9), 329 (43.7), 331 (59.4), 333 (14.8), 213 (8.1), 215 (8.2), 199 (10.4), 171 (11.9), 133 (21.7), 119 (16.9), 91 (25.6), 43 (100). A molecular formula of $C_{15}H_{21}Br_2ClO_2$ by peak matching indicated four degrees of unsaturation.

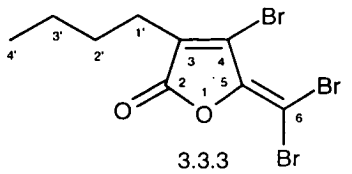
The ^{13}C NMR spectrum showed fifteen carbons corresponding with the formula and the literature values of pacifenol as shown in Table 3.3.4.1. As well, the ^1H NMR spectrum was consistent with the literature data.

Table 3.3.4.1. ^{1}H and ^{13}C NMR data of (3.2.1.46) in $CDCl_3$ and literature values.

No.	(3.2.1.45)		Pacifenol data[77]		Pacifenol data[78]	
	^{1}H, *J* (Hz)	^{13}C	^{1}H, *J* (Hz)	^{13}C	^{1}H, *J* (Hz)	^{13}C
1	4.68 dd, 5.3, 12.5	74.6	4.67 dd, 5.1, 12.6	74.1	4.65 dd, 5.1, 12.5	74.1
2	(eq) 2.69 dd, 5.2, 14.9 (ax) 2.34 m	46.6	(eq) 2.68 dd, 5.1, 14.8 (ax) 2.33 dd, 12.6, 14.8	46.1	(eq) 2.66 dd, 5.1, 14.7 (ax) 2.32 dd, 3.8, 14.4	46.1
3	-	69.6	-	69.0	-	69.1
4	5.45 dd, 3.7, 13.3	60.0	5.43 dd, 4.0, 13.1	59.4	5.41 dd, 3.8, 13.0	59.4
5	(eq) 2.20 d, 12.8 (ax) 2.06 d, 5.0	34.8	(eq) 2.30 dd, 4.0, 10.8 (ax) 2.16 dd, 10.8, 13.1	34.2	(eq) 2.28 dd, 12.5, 14.7 (ax) 2.17 dd, 13.2, 14.4	34.2
6	-	53.9	-	53.3	-	53.3
7	-	77.6	-	77.1	-	77.0
8	6.05 d, 9.8	132.9	6.04 d, 9.8	132.4	6.03 d, 9.7	132.4
9	5.39 d, 9.8	134.9	5.38 d, 9.8	134.3	5.36 d, 9.7	134.3
10	-	100.3	-	99.8	-	99.7
11	-	52.5	-	52.0	-	52.0
12	1.11 s	24.1	1.10 s	23.5	1.08 s	23.5
13	1.29 s	25.2	1.28 s	24.6	1.27 s	24.7
14	1.51 s	25.6	1.50 s	25.1	1.48 s	25.1
15	1.78 s	34.2	1.77 s	33.6	1.76 s	33.6

3.3.5 The structure of 4-bromo-3-butyl-5-(dibromomethylene)-2(5*H*)-furanone (3.3.3).

The fimbrolide (3.3.3), that was previously isolated from red seaweed of the *Delisea* genus,[79, 80, 81, 82] was present only in the sea hare *A. parvula*. Its presence was shown by GCMS of a flash Si gel fraction of a crude extract from the sea hare.

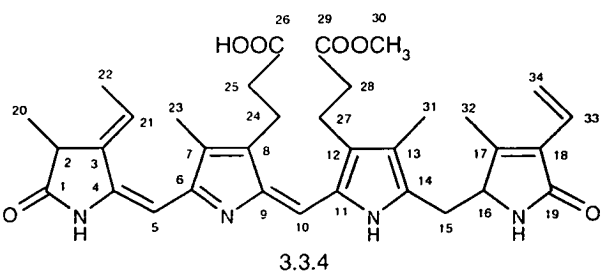


3.3.3

EI mass spectra showed m/z 386 (M^+, 7.8), 388 (24.1), 390 (24.0), 392 (8.6), 357 (1.7), 359 (6.0), 361 (6.0), 363 (1.7), 344 (15.5), 346 (46.6), 348 (43.1), 350 (14.7), 330 (1.4), 332 (3.9), 334 (3.7), 336 (1.3), 307 (49.1), 309 (100), 311 (49.1), 287 (1.7), 289 (5.7), 291 (5.9), 293 (1.7), 265 (22.8), 267 (44.8), 269 (21.6), 237 (4.5), 239 (8.6), 241 (4.5), 227 (7.8), 229 (8.6), 105 (19.8), 91 (34.5), 81 (35.3), 41 (47.4). The mass spectrum was consistent with the literature.[79]

3.3.6 The structure of aplysioviolin (3.3.4).

Aplysioviolin was previously isolated from the sea hare *Aplysia limacine* and assigned structure (3.1.6.4).[24] In this study aplysioviolin has been isolated as a purple solid film, 0.014% yield (based on the sea hare dry weight) and its physical properties were identical with those in the literature. However, a revised structure of aplysioviolin has been proposed here as (3.3.4).

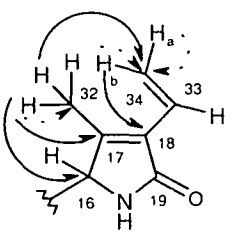


3.3.4

The HRLSIMS gave $C_{34}H_{41}N_4O_6$, observed as 601.30080 $[M+H]^+$, the same as the ESI LCMS result of 601 for $[M+H]^+$, which corresponds to a molecular formula of $C_{34}H_{40}N_4O_6$, indicating seventeen degrees of unsaturation.

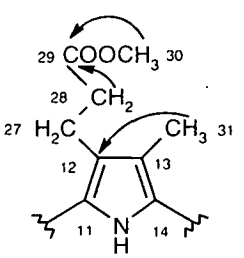
NMR experiments were used to partially establish the structure as follows. Firstly, the only olefinic methylene carbon (C-34) was detected at 119.3 ppm. HMQC showed one bond connections of the carbon (C-34) at 119.3 ppm to both protons (H-34a) at 5.31 ppm and (H-34b) at 6.03 ppm. The proton (H-34a) was *cis* to a methine proton (H-33) at 6.51 ppm because of its lower coupling constant (J = 10 Hz). Proton H-34b was *trans* to the proton (H-33) as it showed a higher coupling constant (J = 18.4 Hz).

In addition, the *trans* proton (H-34b) at 6.03 ppm was connected to a quaternary carbon (C-18) at 137.4 ppm by HMBC. The carbon (C-34) was linked by HMBC to the methyl protons $(H\text{-}32)_3$ at 2.09 ppm; these protons were also connected to a quaternary carbon (C-17) at 128.9 ppm and to a methine carbon (C-16) at 61.0 ppm by HMBC. HMQC showed a link between $(H\text{-}32)_3$ at 2.09 ppm to C-32 at 12.8 ppm. Moreover, a methine proton (H-16) at 4.42 ppm connected to the carbon (C-16) at 61.0 ppm by HMQC. These data allowed ring A to be defined.

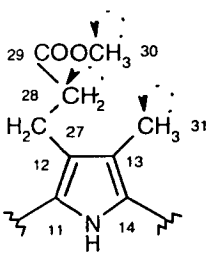


ring A

Ring B was established as follows. Connectivities by HMBC of methyl protons $(H\text{-}30)_3$ at 3.59 ppm to an ester carbonyl (C-29) at 175.1 ppm and of methylene protons $(H\text{-}28)_2$ at 2.52 ppm to the carbonyl carbon (C-29) were evident. A linkage of methyl protons $(H\text{-}31)_3$ at 1.99 ppm to a quaternary carbon (C-12) at 124.7 ppm was displayed. HMQC connectivities of C-30 at 52.2 ppm to H-30 at 3.59 ppm, C-28 at 36.2 ppm to H-28 at 2.52 ppm, and C-31 at 15.1 ppm to H-31 at 1.99 ppm were observed.



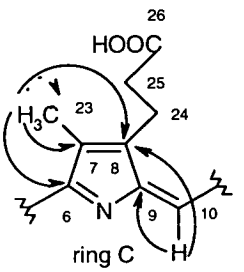
ring B



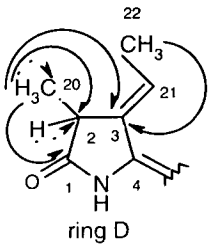
ring B

Ring C was established as follows. HMBC displayed connectivities of methyl protons (H-23) at 2.05 ppm to C-7 at 129.3 ppm, to C-8 at 145.1 ppm and to C-6 at 156.7

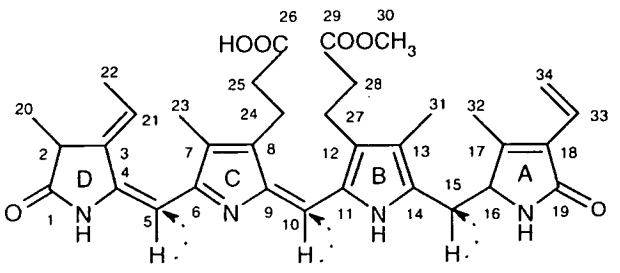
ppm. An olefinic methine proton (H-10) at 6.81 ppm was connected to C-9 at 127.1 ppm and to C-8 at 145.1 ppm by HMBC. HMQC also showed a connection of H-23 at 2.05 ppm to C-23 at 9.7 ppm.



Finally, ring D was established as follows. Connectivities between methyl protons $(H\text{-}20)_3$ at 1.38 ppm to C-2 at 39.5 ppm, protons $(H\text{-}20)_3$ to a carbonyl carbon (C-1) at 181.8 ppm, and protons $(H\text{-}20)_3$ to C-3 at 137.4 ppm were established by HMBC. In addition, methyl protons $(H\text{-}22)_3$ at 1.93 ppm were linked to the carbon (C-3) by HMBC. The methine proton (H-2) at 3.22 ppm was connected to the carbon (C-2) at 39.5 ppm by HMQC. Similarly, the methyl protons $(H\text{-}20)_3$ at 1.38 ppm were linked to the carbon (C-20) at 16.5 ppm by HMQC.



Unfortunately, the connectivity between rings A, B, C, and D could not be established by HMBC. The carbon (C-5) at 88.5 ppm was linked to H-5 at 5.96 ppm, the carbon (C-10) at 115.0 ppm was joined to H-10 at 6.81, and the carbon (C-15) at 21.9 ppm was connected to $(H\text{-}15)_2$ at 2.91 ppm by HMQC. By comparison to linear tetrapyrroles, a structure (3.3.4) is shown below.



3.3.4

At this point, NMR experiments could not distinguish where the methyl group (C-30) was as structure (3.3.4) or (3.3.6). In other words, once the partial structures of ring A to D were established, the pendant groups could only be placed as shown in structure (3.3.4) or (3.3.6). In addition, a complexity of tautomers should be addressed. For examples, structures (3.3.4) and (3.3.5) are tautomers. Similarly, structures (3.3.6) and (3.3.7) are tautomers. Therefore, which of these structures (eg. 3.3.4-3.3.7) was the structure of aplysioviolin?

^{1}H NMR data of the other linear tetrapyrroles, phycocyanobilin dimethyl ester and mesobiliverdin dimethyl ester were previously assigned.[25,26] In addition, ^{1}H and ^{13}C NMR data of the synthetic racemic dimethyl ester phycoerythrobilin (3.1.6.13) were also assigned.[31]

^{1}H and ^{13}C NMR (Figure 3.3.6.1- 3.3.6.2, Table 3.3.6.1), as well as HMQC (Figure 3.3.6.3) and HMBC experiments (Figure 3.3.6.4) of aplysioviolin (3.3.4) were established.

The molecular modelling tool HyperChem[83] was used to calculate the geometry optimization total energy of the molecule. The result using HyperChem showed that the geometric optimization total energy of the structures (3.3.4, 3.3.5, 3.3.6, and 3.3.7) were not significantly different having values of 61.4262, 60.2467, 61.9376, and 59.8090 Kcal.mol^{-1}, respectively. Therefore, this method was not useful in distinguishing between tautomers.

Table 3.3.6.1. ^{1}H and ^{13}C NMR data of (3.3.4) in $CDCl_3$ and CD_3OD.

No.	(3.3.4.1) in $CDCl_3$			(3.3.4.1) in CD_3OD		
	^{1}H, *J* (Hz)	^{13}C, DEPT	HMBC	^{1}H, *J* (Hz)	^{13}C, DEPT	HMBC
1	-	179.1, C	2, 20	-	181.8, C	20
2	3.25 m	38.2, CH	21	3.22 br, m	39.5, CH	20
3	-	136.8, C	5, 20, 22	-	137.4, C	20, 22
4	-	127.2, C	22	-	127.1, C	21
5	5.70 s	85.8, CH	-	5.96 s	88.5, CH	-
6	-	127.2, C	23	-	156.7, C	-
7	-	120.8, C	23, 24	-	129.3, C	23
8	-	145.2, C	24	-	145.1, C	23
9	-	127.2, C	-	-	127.1, C	10
10	6.80 s	116.1, CH	-	6.81 s	115.0, CH	-
11	-	144.8, C	10	-	147.9, C	-
12	-	120.8, C	-	-	128.9, C	31
13	-	145.2, C	27, 31	-	156.7, C	31
14	-	144.8, C	-	-	127.1, C	-
15	2.60 br, m	35.0, CH_2	-	2.91 br, m	21.9, CH_2	-
16	4.33 br, m	59.9, CH	32	4.42 br, dt	61.0, CH	31
17	-	154.0, C	-	-	128.9, C	-
18	-	128.5, C	34a, 34b	-	137.4, C	34
19	-	178.0, C	15	-	179.0, C	-
20	1.40 d	16.3, CH_3	2	1.38 d, 6.8	16.5, CH_3	-
21	6.30 m	123.4, CH	-	6.41 q, 5.6, 11.2, 16.8	127.1, CH	-
22	1.94 s	9.2, CH_3	-	1.93 d, 7.6	9.3, CH_3	-
23	1.94 s	15.1, CH_3	-	2.05 s	9.7, CH_3	-
24	2.80 m	20.9, CH_2	-	2.91 m	21.9, CH_2	25
25	2.40 m	29.4, CH_2	-	2.45 t, 7.2	30.3, CH_2	-
26	-	173.2, C	25	-	175.0, C	-
27	2.80 m	19.8, CH_2	-	2.91 m	21.0, CH_2	-
28	2.40 m	29.8, CH_2	-	2.52 t, 7.2	36.2, CH_2	-
29	-	173.5, C	27, 28, 30	-	175.1, C	28, 30
30	3.60 s	51.9, CH_3	-	3.59 s	52.2, CH_3	-
31	1.96 s	12.6, CH_3	-	1.99 s	15.1, CH_3	-
32	2.07 s	9.6, CH_3	-	2.09 s	12.8, CH_3	-
33	6.04 dd	125.8, CH	34	6.51 d, 7.6	124.7, CH	32
34	5.32 dd 6.15 dd	119.4, CH_2	-	5.31 dd, 10 6.03 dd, 18.4	119.3, CH_2	32

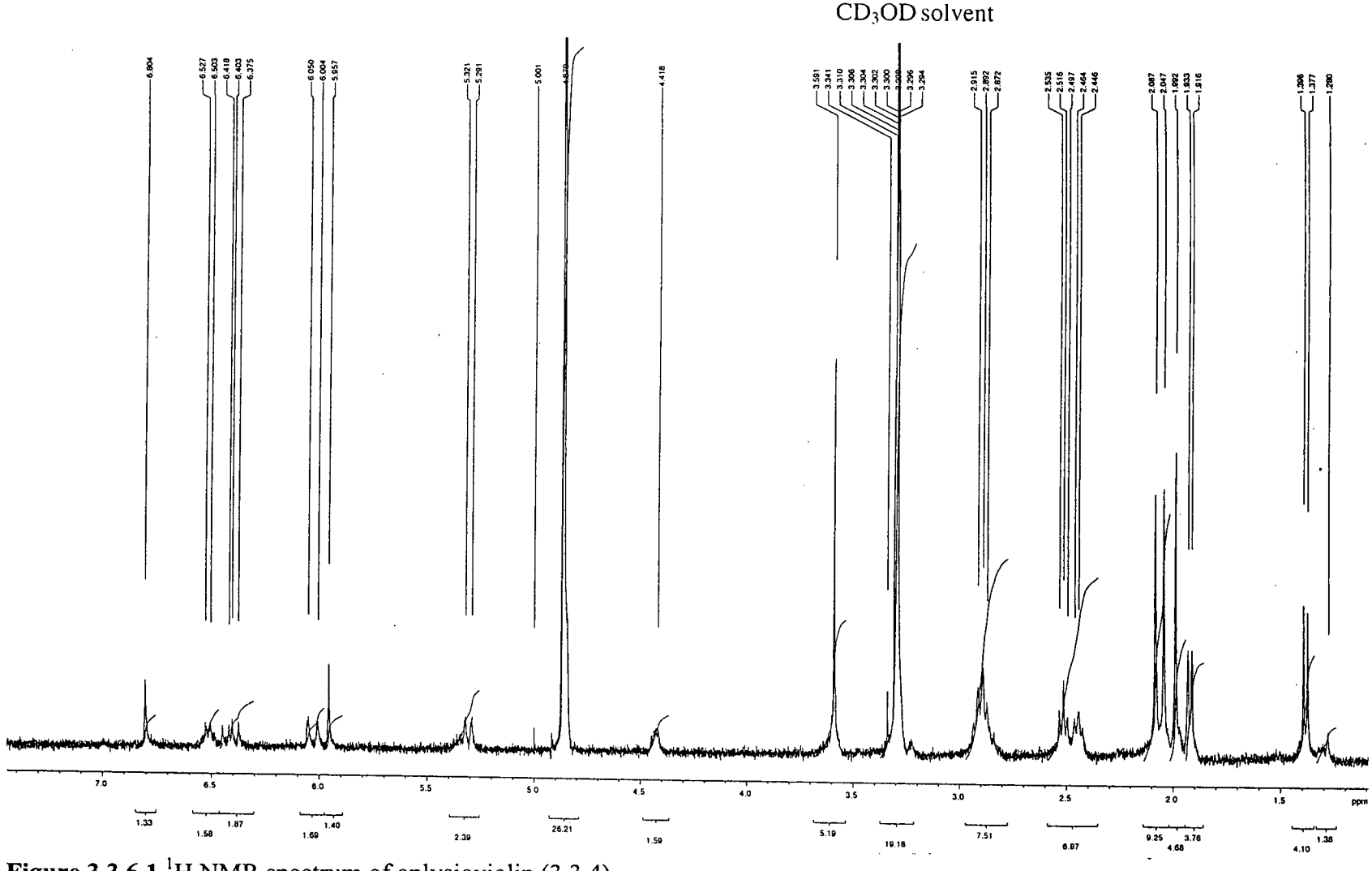


Figure 3.3.6.1 ^{1}H NMR spectrum of aplysioviolin (3.3.4).

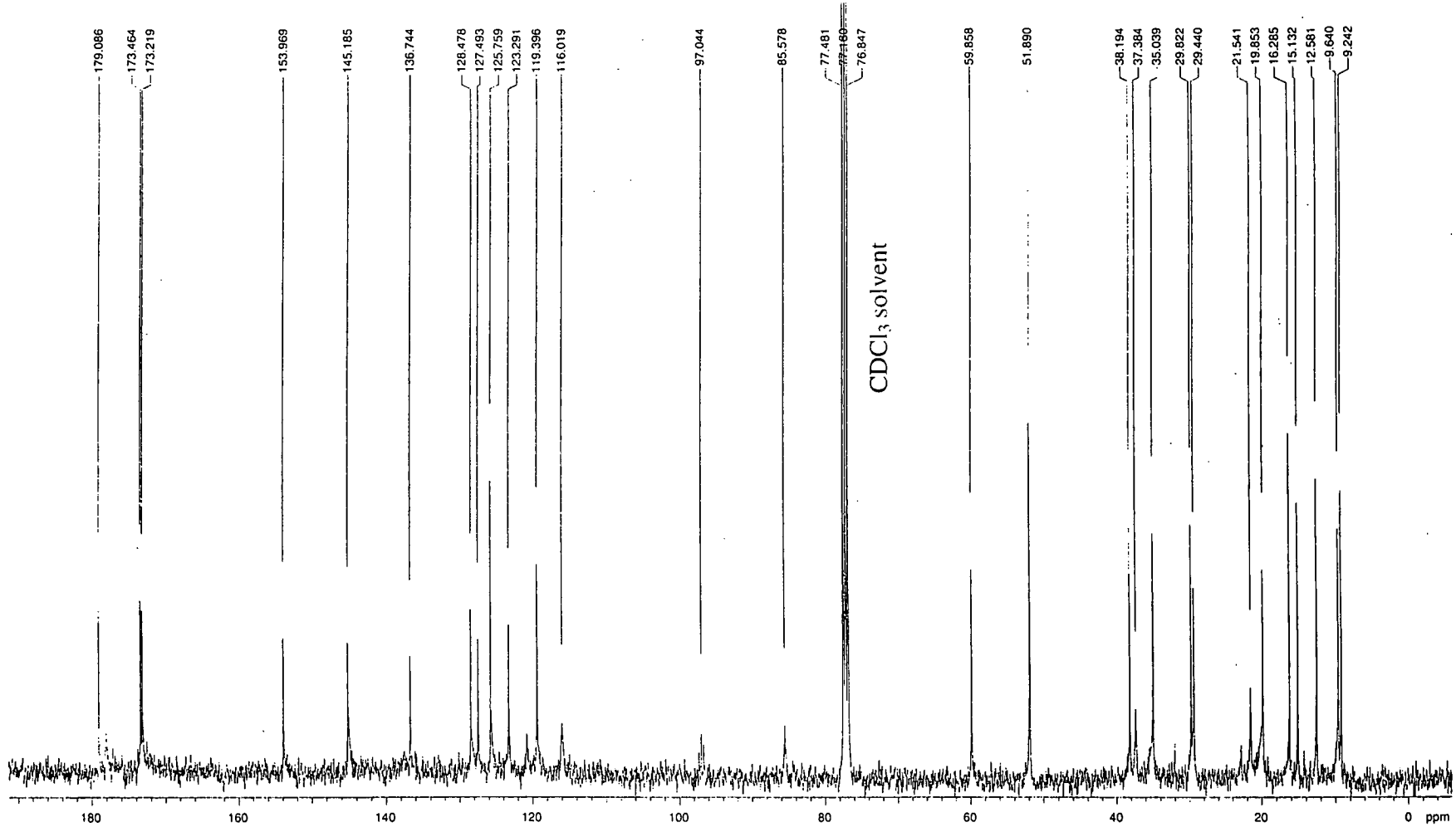


Figure 3.3.6.2 ^{13}C NMR spectrum of aplysioviolin (3.3.4).

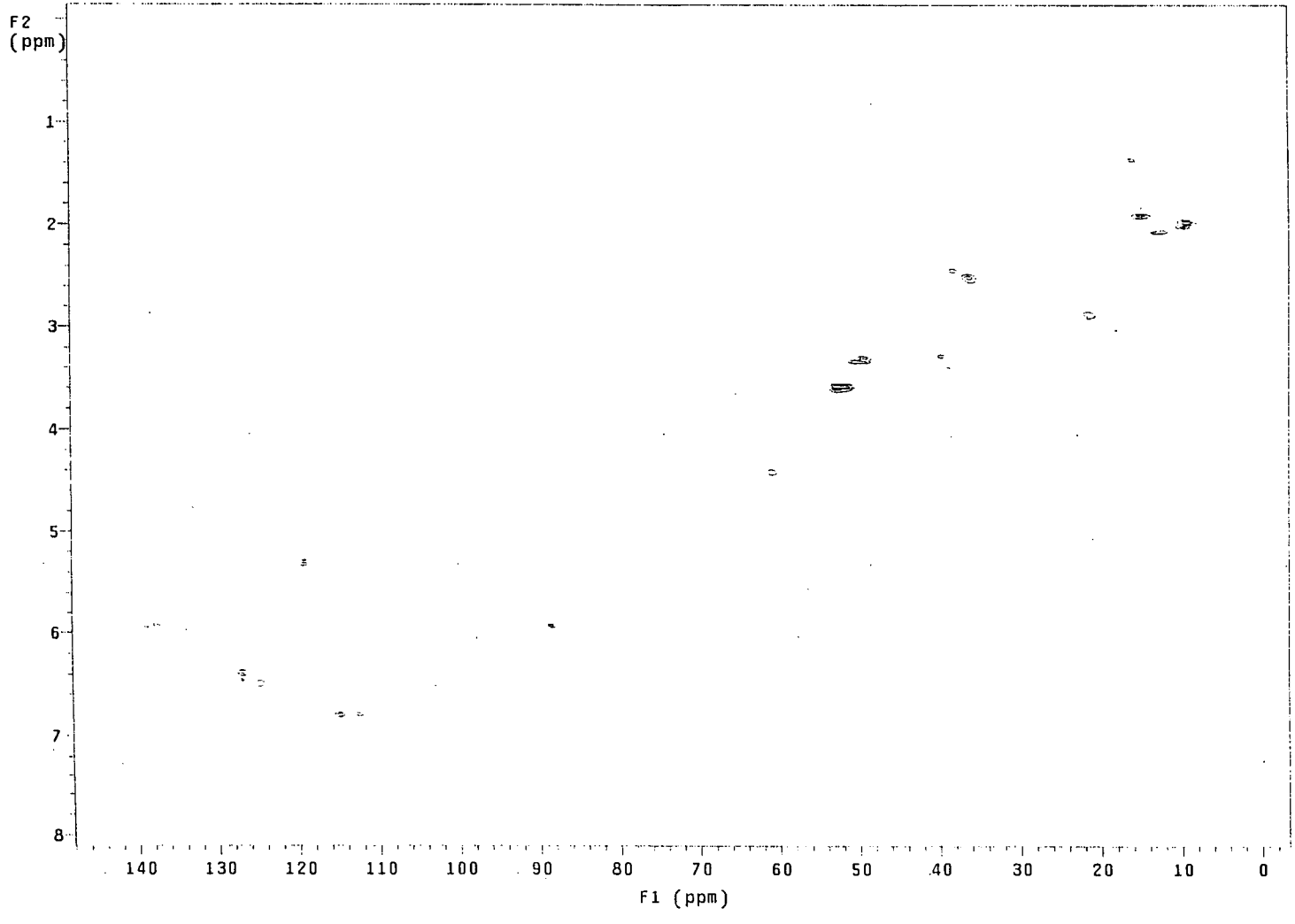


Figure 3.3.6.3 HMQC experiment of aplysioviolin (3.3.4).

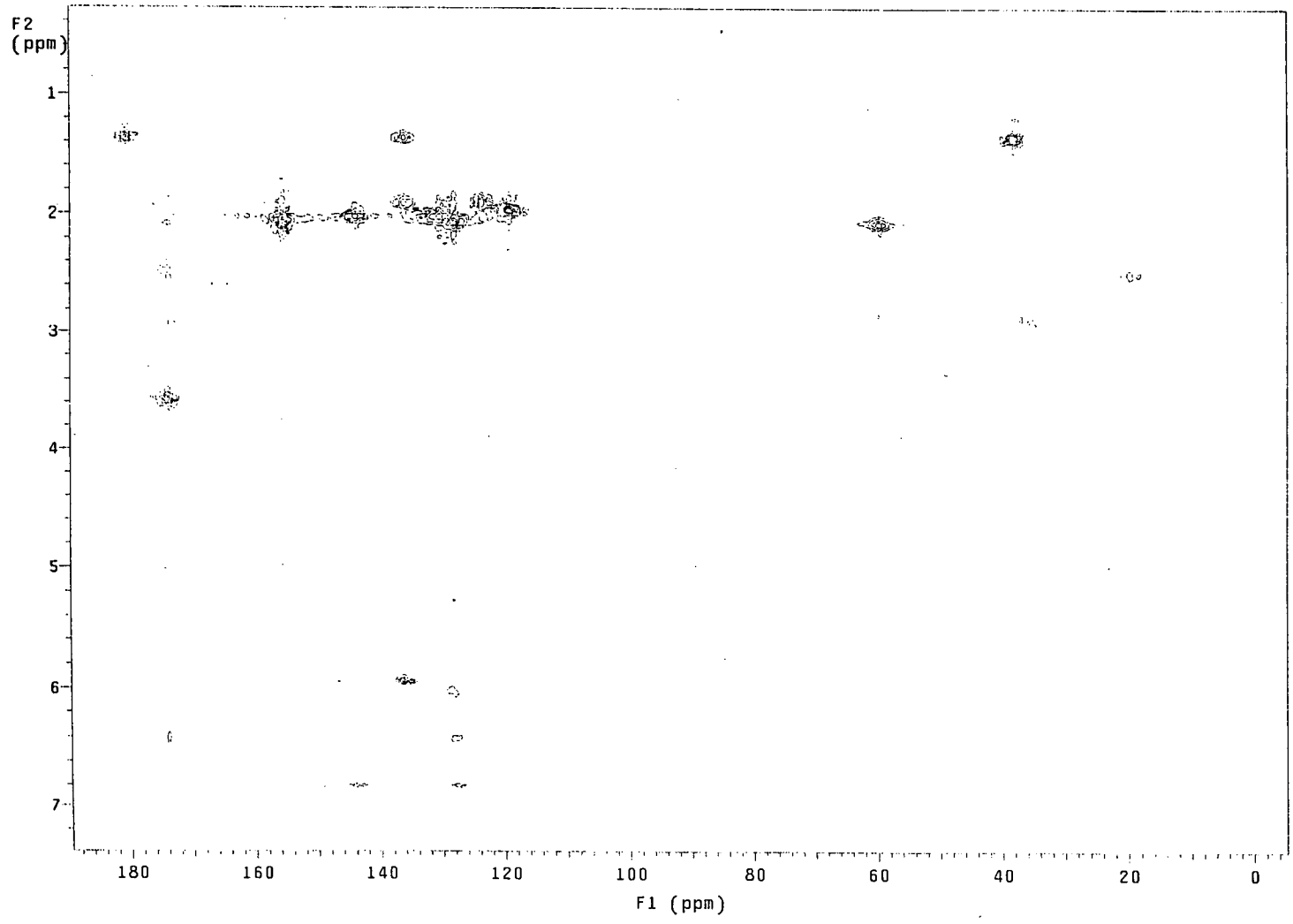
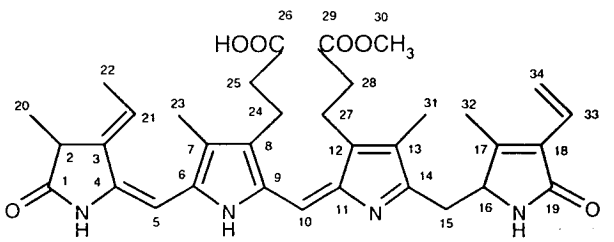


Figure 3.3.6.4 HMBC experiment of aplysioviolin (3.3.4).

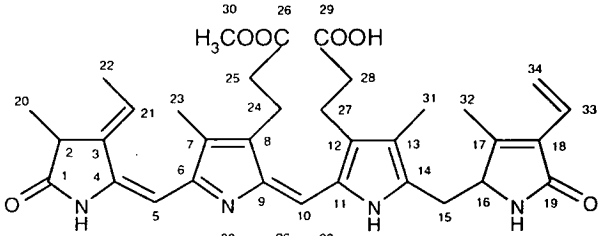
In order to overcome the problem, MS/MS spectrometry was used. The principle is that specific ions or "parent ions" are separated in the first mass spectrometer and passed into the collision chamber, one at a time. Then "daughter ions" in the collision chamber are formed by collision with an introduced gas (helium). Next these ions are passed into the second mass spectrometer, where a daughter-ion spectrum is produced. A mass spectrum of each selected ion of the first spectrum is thus displayed, the second one is produced, and so on as MS/MS spectrometry.[73]

Tandem mass spectrometry (MS/MS) was performed on the isolated purple pigment and gave a series of parent ions to daughter ions one at a time as follows: 601 (MH) → 478 (MH-123) → 297 (MH-123-181) → 237 (MH-123-181-60) → 222 (MH-123-181-60-15).

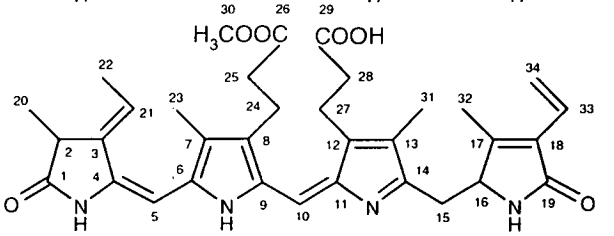
Structures (3.3.4-3.3.7) were analysed to see which would best fit this MS/MS result (Figure 3.3.6.1-3.3.6.5).



3.3.5

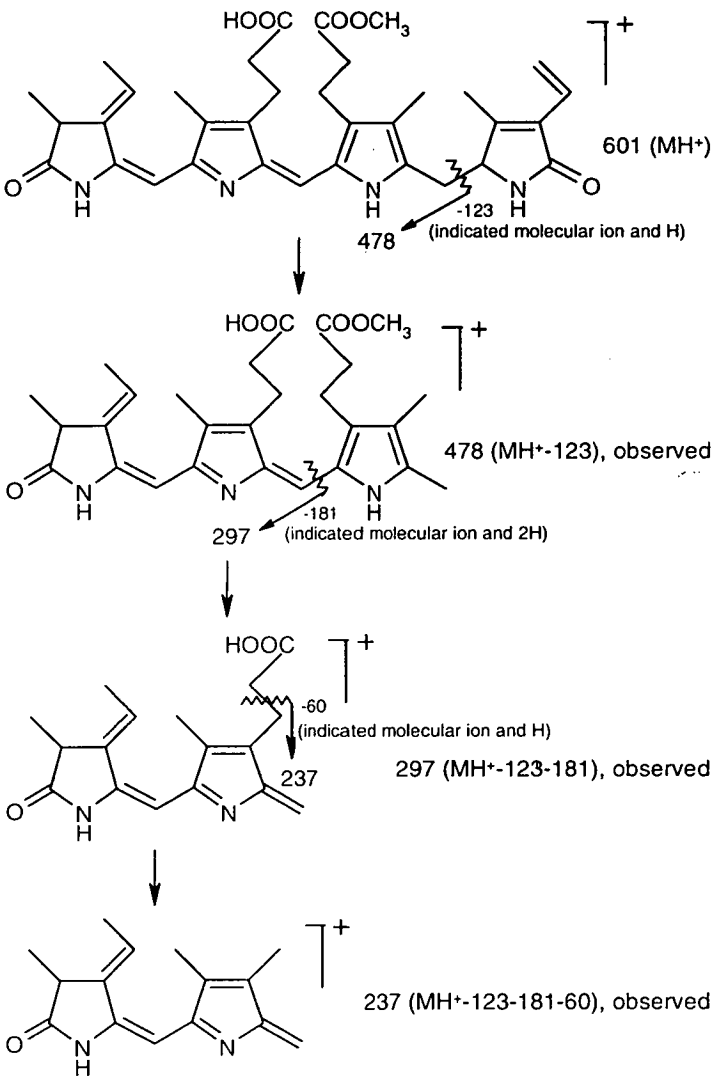


3.3.6

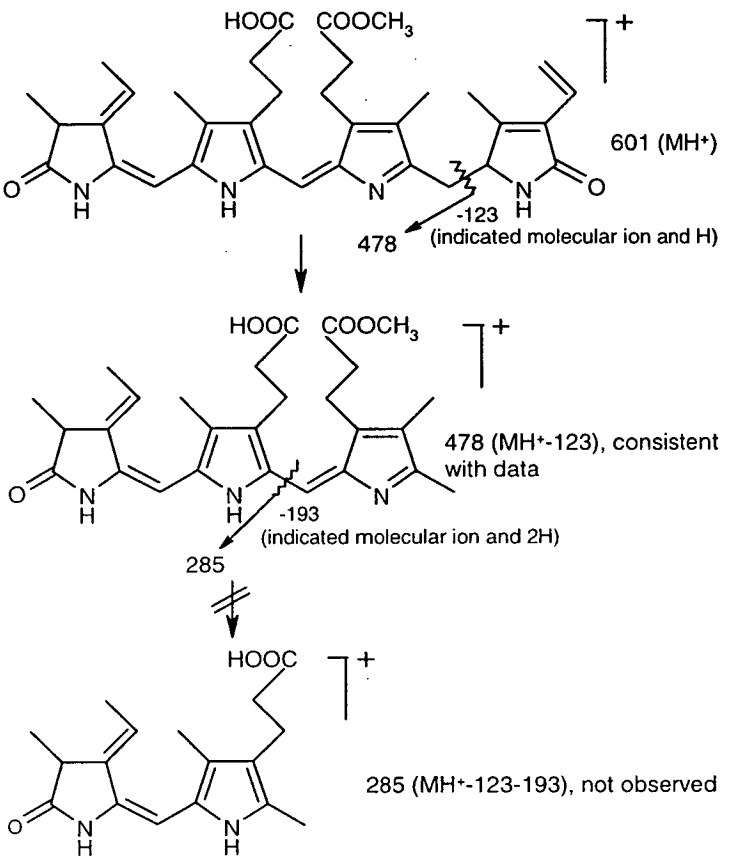


3.3.7

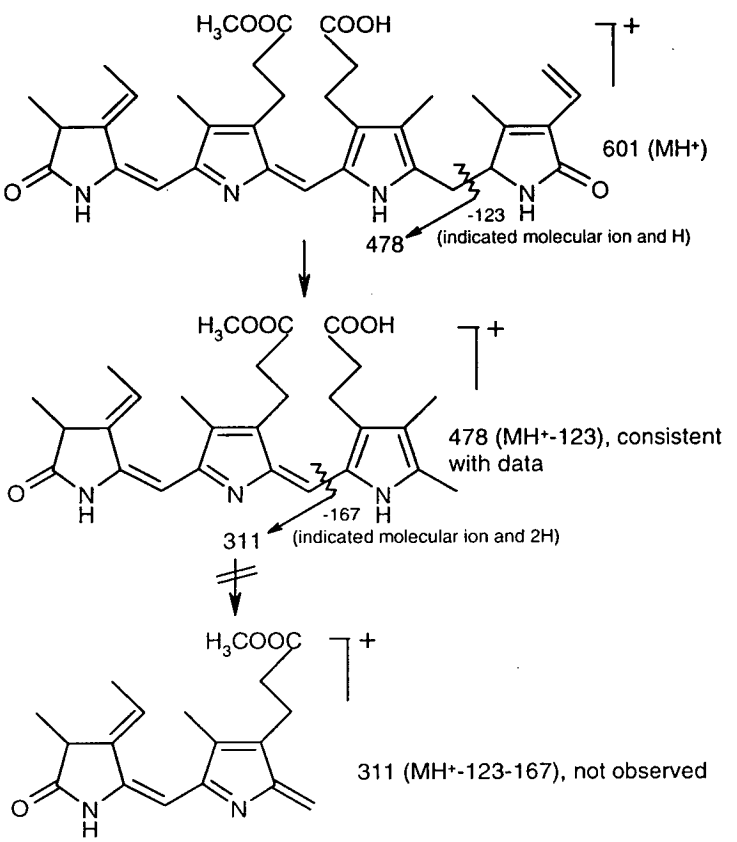
The proposed MS/MS fragmentation (Scheme 3.3.6.1) of structure (3.3.4) was consistent with the MS/MS data (Figure 3.3.6.5-3.3.6.9), which was contrary to the other proposed fragmentations (Scheme 3.3.6.2-3.3.6.4) of the other structures (3.3.5-3.3.7). Structure (3.3.7) had been previously proposed for aplysioviolin (3.1.6.4). Therefore, the ink pigment aplysioviolin needs a structural revision to compound (3.3.4). However, more supporting data are needed to establish which is the preferred tautomer.



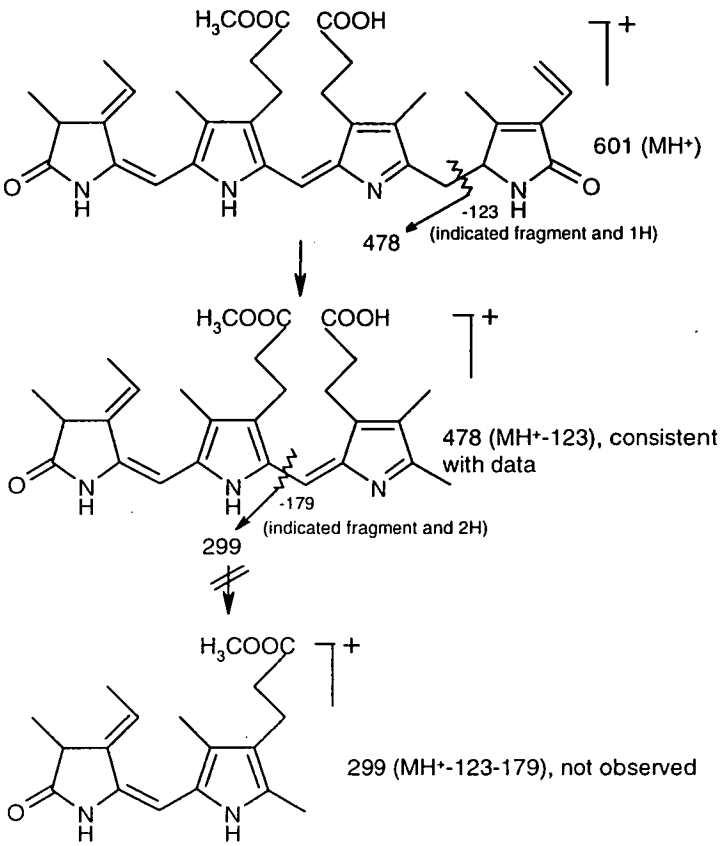
Scheme 3.3.6.1 MS/MS fragmentation of (3.3.4), location of charges and extra H(s) not shown.



Scheme 3.3.6.2 MS/MS fragmentation of (3.3.5), location of charges and extra H(s) not shown.



Scheme 3.3.6.3 MS/MS fragmentation of (3.3.6), location of charges and extra H(s) not shown.



Scheme 3.3.6.4 MS/MS fragmentation of (3.3.7), location of charges and extra H(s) not shown.

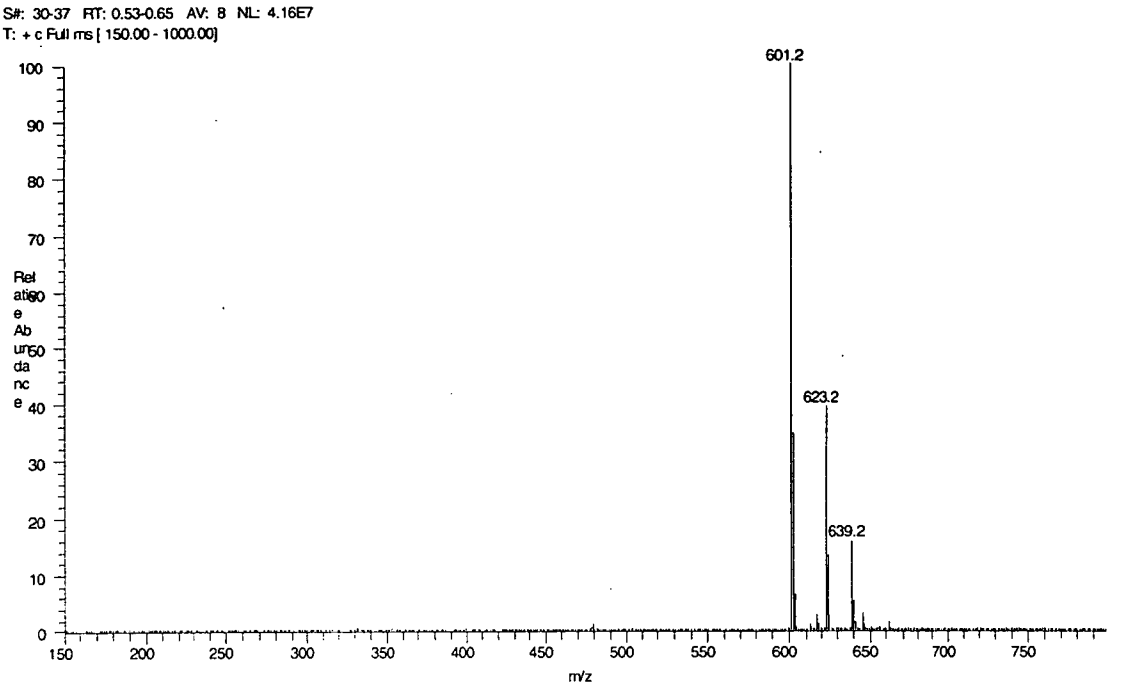


Figure 3.3.6.5 MS/MS of aplysioviolin with a parent ion 601 (M^++Na at 623, M^++K at 639).

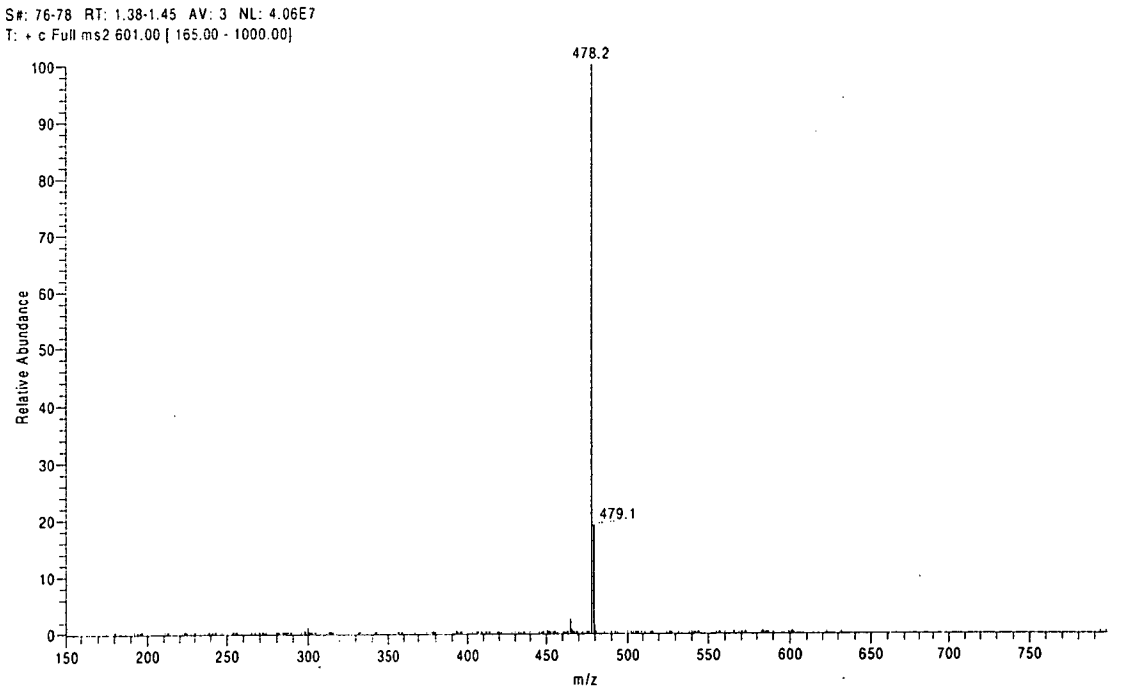


Figure 3.3.6.6 MS/MS of aplysioviolin from the ion 601 to 478.

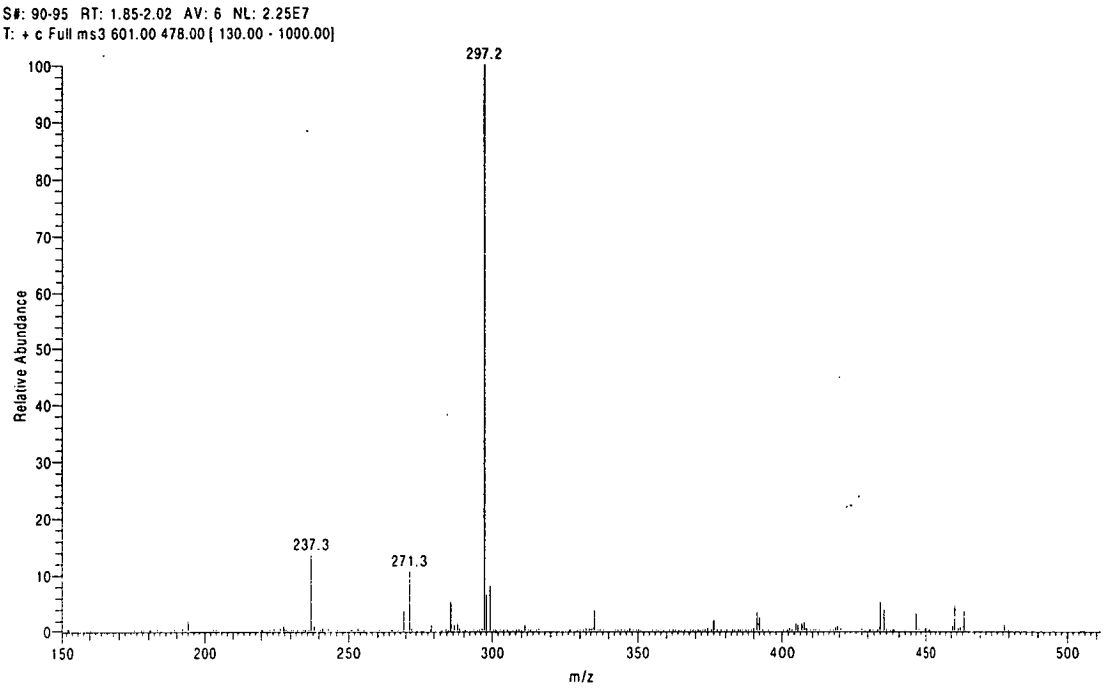


Figure 3.3.6.7 MS/MS of aplysioviolin from the ion 478 to 297.

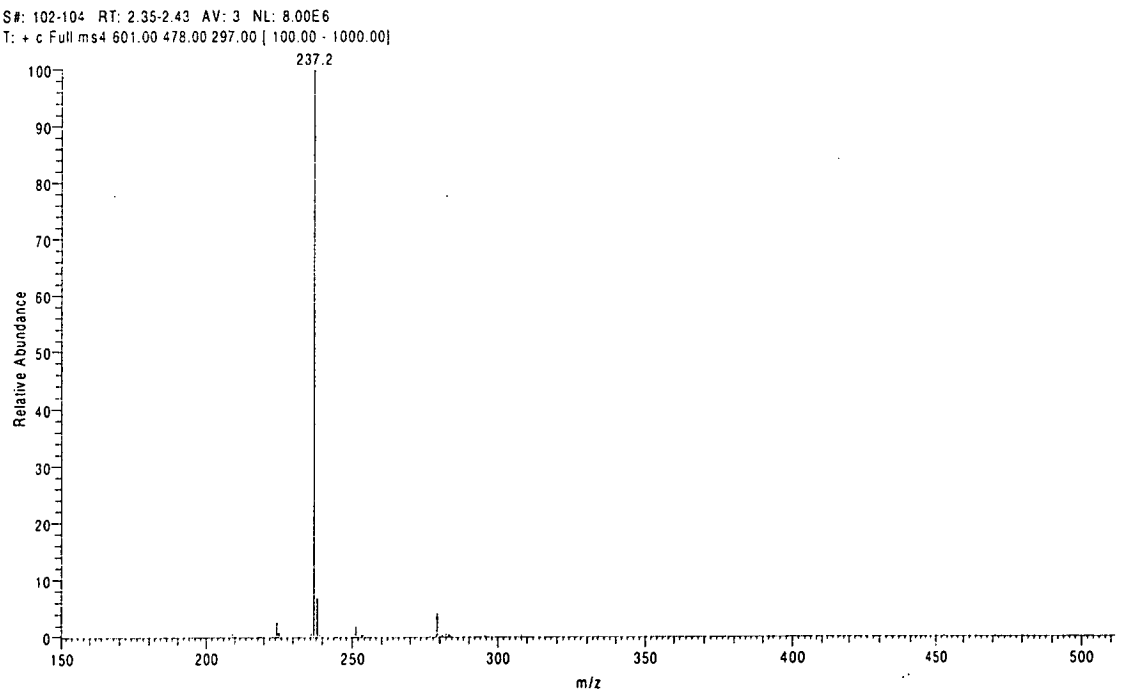


Figure 3.3.6.8 MS/MS of aplysioviolin from the ion 297 to 237.

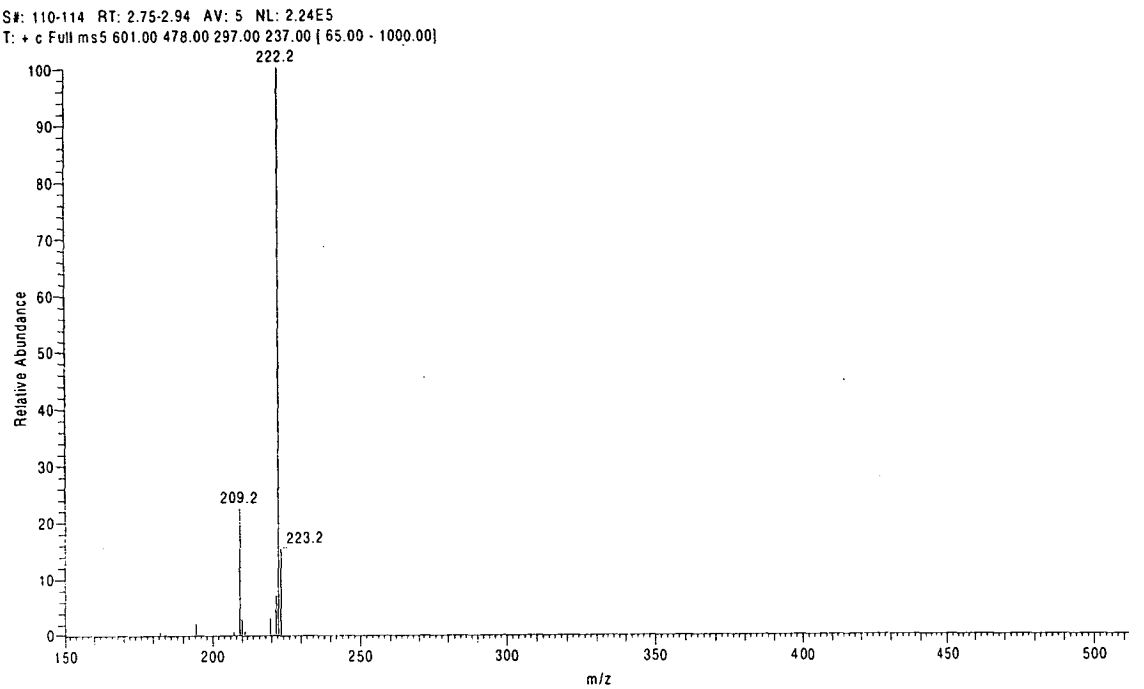
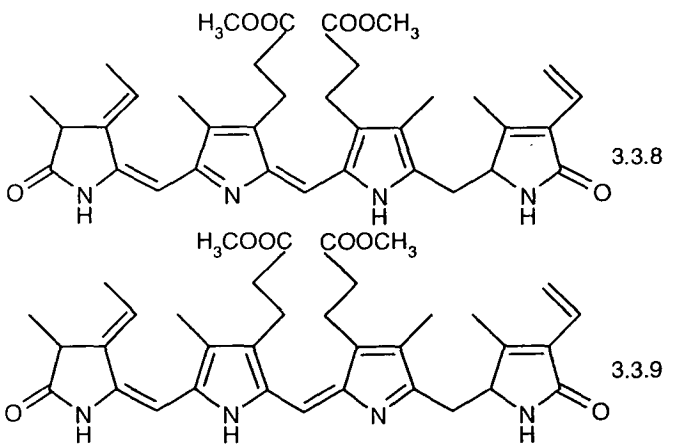
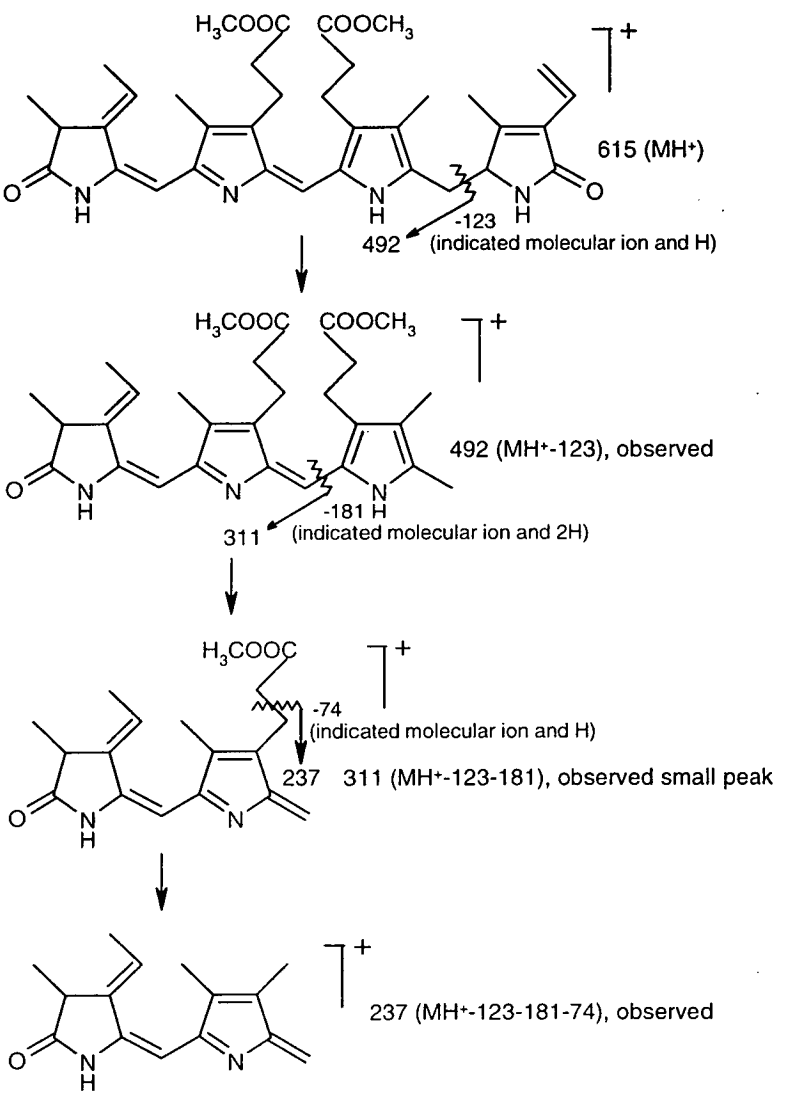


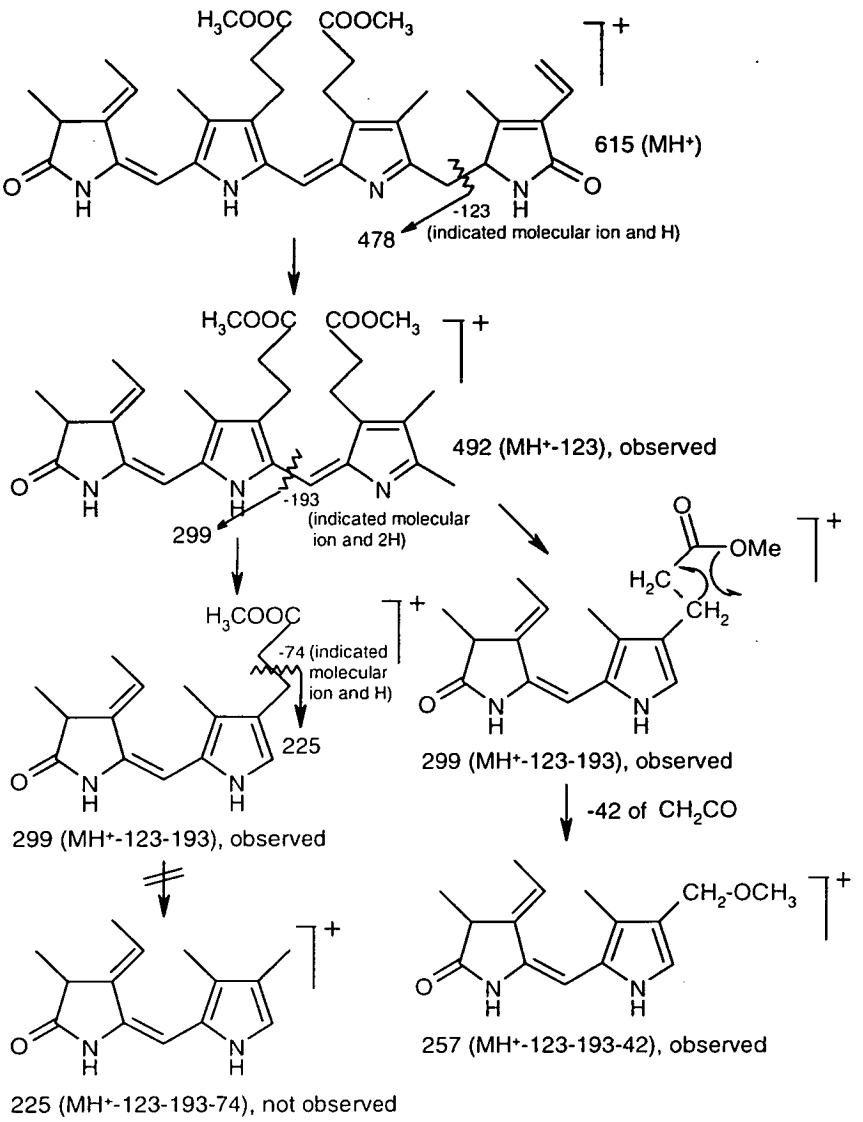
Figure 3.3.6.9 MS/MS of aplysioviolin from the ion 297 to 222.

The isolated purple pigment from this study was derivatized with diazomethane to form a dimethyl ester derivative compound and MS/MS experiments (Figure 3.3.6.10-3.3.6.14) were performed. A series of parent ions to daughter ions were recorded as followed: 615 (MH) → 492 (MH-123) → 299 (MH-123-193) → 257 (MH-123-193-42) and also the selected daughter ion of 492 which gave a small peak of 311 (MH-181) → 237 (MH-181-74) were detected. Two tautomers (3.3.8-3.3.9) of the synthesised dimethyl ester were considered for the proposed MS/MS fragmentation (Scheme 3.3.6.5-3.3.6.6). Both of them are needed to account for the data so therefore the methyl affected the MS/MS. This assumption is based on there being no tautomerisation during the fragmentation process.





Scheme 3.3.6.5 MS/MS fragmentation of (3.3.8), location of charges and extra H(s) not shown.



Scheme 3.3.6.6 MS/MS fragmentation of (3.3.9), location of charges and extra H(s) not shown.

METHYLATED VERSION

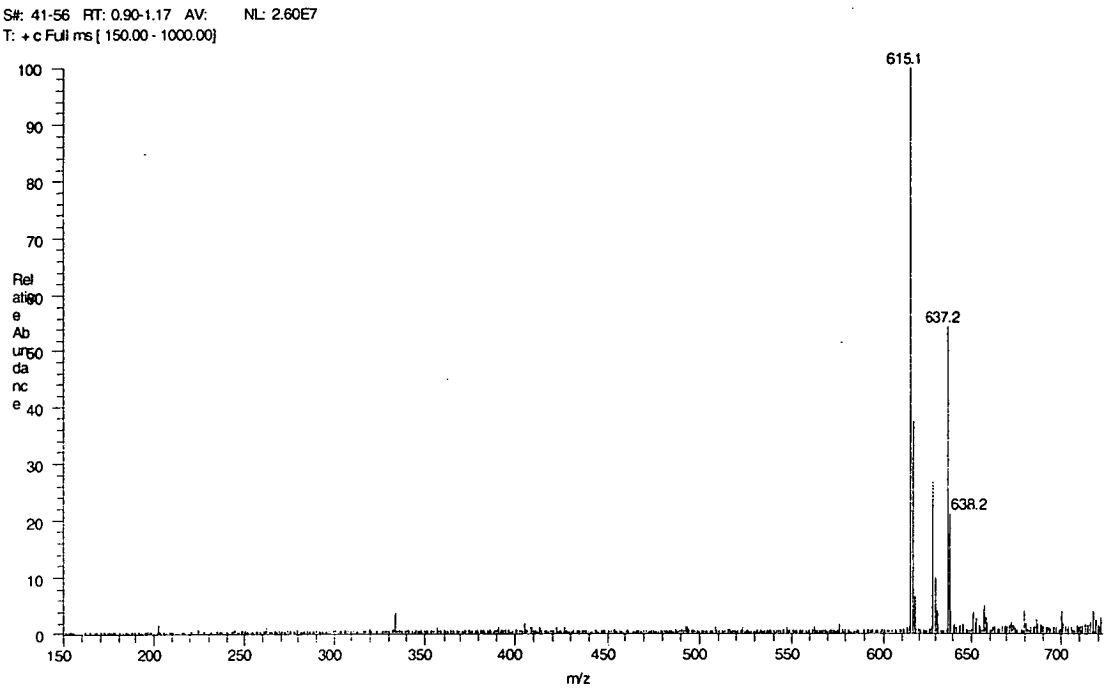


Figure 3.3.6.10 MS/MS of the methylated aplysioviolin with a parent ion 615 (M^++ Na at 637).

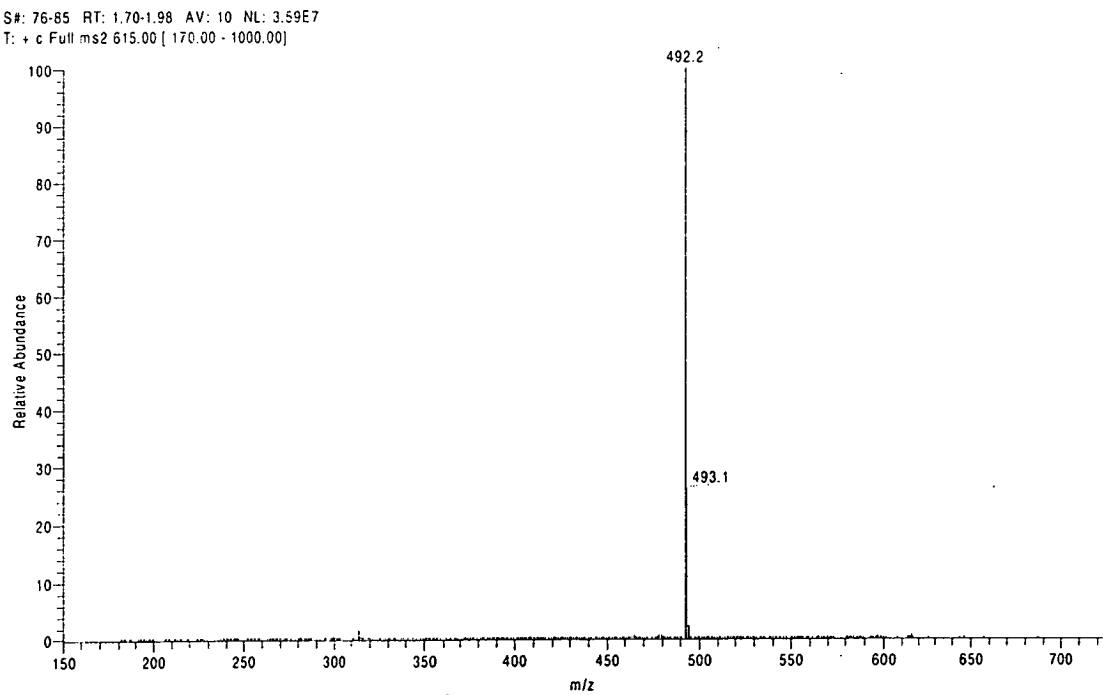


Figure 3.3.6.11 MS/MS of the methylated aplysioviolin from the ion 615 to 492.

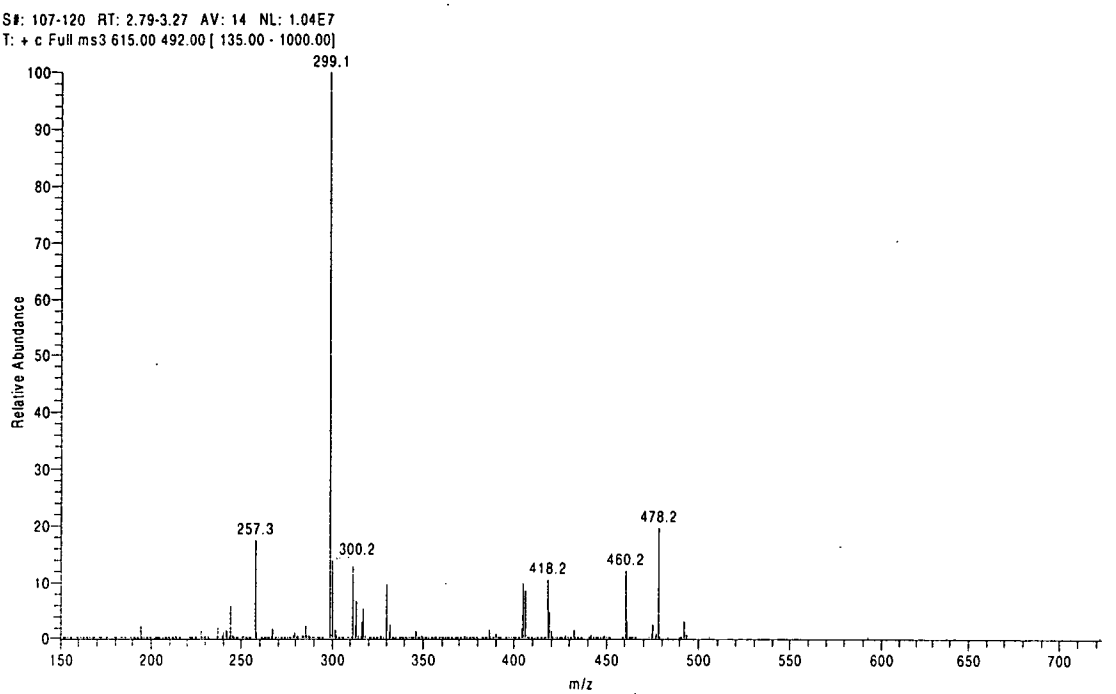


Figure 3.3.6.12 MS/MS of the methylated aplysioviolin from the ion 492 to 299.

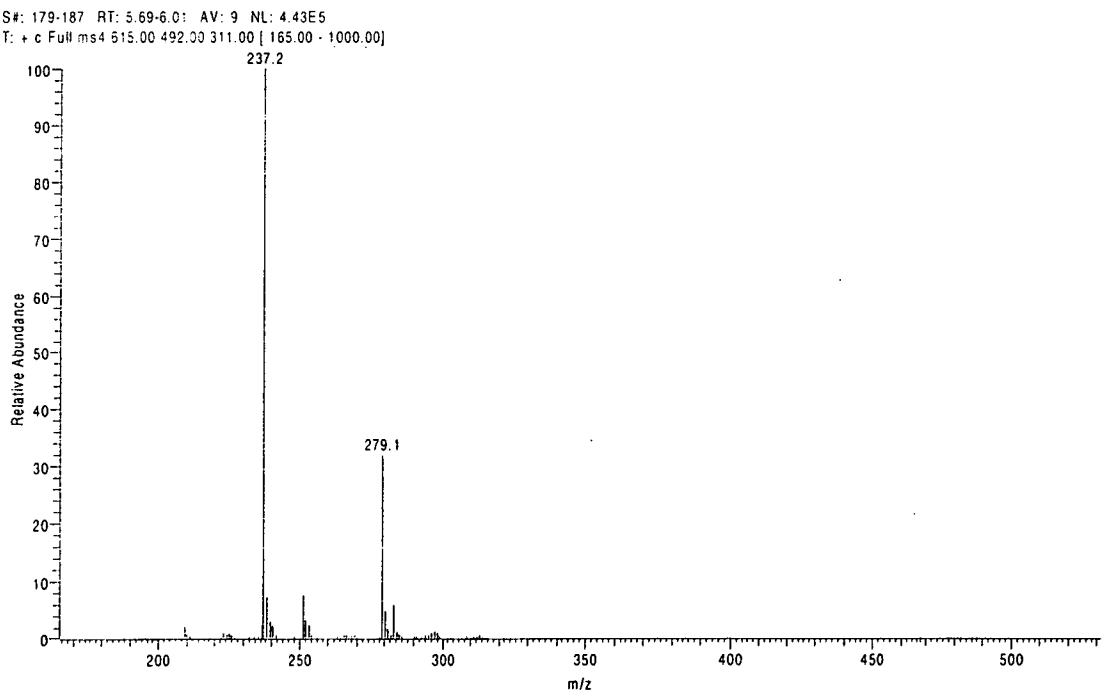


Figure 3.3.6.13 MS/MS of the methylated aplysioviolin from the ion 311 to 237.

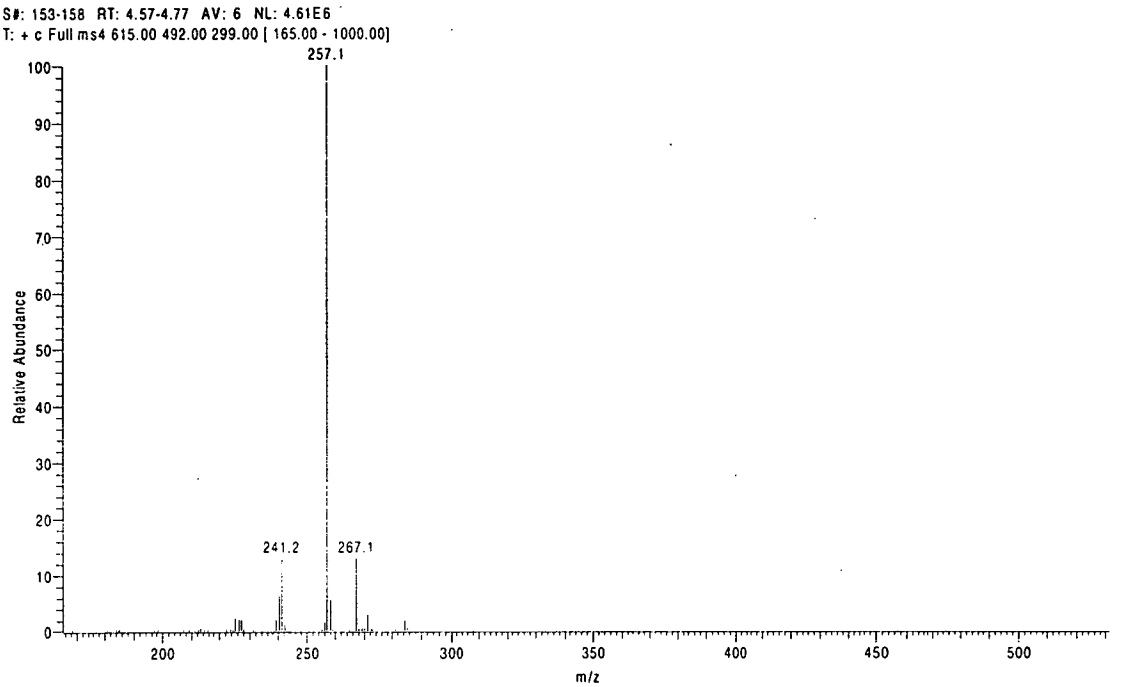
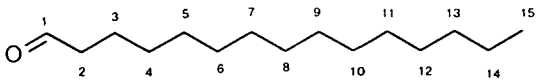


Figure 3.3.6.14 MS/MS of the methylated aplysioviolin from the ion 299 to 257.

3.3.7 The structure of pentadecanal (3.3.10).

Pentadecanal (3.3.10) was separated as a minor component (0.005% yield base on the seaweed dry weight) from the red seaweed *L. filiformis*.



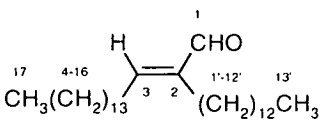
3.3.10

The GCMS spectrum of the isolated pentadecanal (3.3.10) revealed m/z 226 (M^+, 0.4), 208 (0.7), 198 (0.4), 193 (0.2), 182 (1.8), 165 (0.7), 152 (1.8), 138 (3.2), 124 (5.3), 110 (9.6), 96 (14.4), 82 (56.1), 67 (36.8), 57 (82.4), 55 (63.8), 43 (100), 41 (98.2). The structure was consistent with the GCMS database.[84]

The ^{1}H NMR spectrum of the isolated pentadecanal (3.3.10) supported an aldehyde proton and the rest of the structure as follows: δ 0.88 (CH_3, t), 1.26 ((CH_2)$_9$(CH_2)$_3$$CH_2$ CHO, s), 1.57 (CH_2)$_3$$CH_2$CHO, s), 2.42 ($CH_2$CHO, dt, J=1.9, 5.4 Hz), 9.77 (CHO, t, J=1.9 Hz).

3.3.8 The structure of (*E*)-2-tridecyl-2-heptadec-2-enal (3.3.11).

(*E*)-2-tridecyl-2-heptadec-2-enal (3.3.11) was separated as a minor component (0.010% dry wt) from the red seaweed *L. filiformis*. Previously (*E*)-2-tridecyl-2-heptadecenal (3.3.11)[85] had been isolated from a red alga *Laurencia* species and confirmed by synthesis from pentadecanal (3.3.10). Since compound (3.3.11) can be prepared in the laboratory from pentadecanal it is possible that it may not be a genuine secondary metabolite of *L. filiformis*. However, a GCMS analysis of an extract that was carefully prepared to minimize the occurrence of an aldol reaction, revealed that compound (3.3.11) was present making it less likely to be an artifact.



3.3.11

A GCMS spectrum showed m/z 434 (M^+, 44.7), 416 (0.6), 402 (1.5), 377 (0.2), 349 (0.2), 321 (0.6), 293 (2.3), 265 (7.6), 251 (11.2), 237 (13.6), 193 (2.3), 169 (1.5), 149 (3.0), 135 (7.6), 98 (29.5), 83 (34.8), 69 (32.6), 57 (50.0), 55 (55.8), 43 (100), 41 (56.8).

The ^{1}H NMR spectrum supported an aldehyde moiety and the rest of the structure as follows: δ 0.88 (2 -$\mathbf{CH_3}$, t, *J*=6.3 Hz), 1.26 and 1.56 ($\mathbf{(CH_2)_{12}CH_2CH_3}$ and $\mathbf{(CH_2)_{11}CH_2CH_3}$, s), 2.0 and 2.4 m, 2 -$\mathbf{CH_2CH_3}$, s), 6.44 (**CH**CHO, t, *J*=7.6 Hz), 9.36 (**CHO**, s).The structure was consistent with the ^{1}H NMR literature data.[87]

3.4 Conclusion.

In this study, compound (3.3.1), which was separated from both the sea hare *A. parvula* and the red seaweed *L. filiformis* (as 0.073 and 0.230% yield base on the sea hare and the seaweed dry weight, respectively), is 5-acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene with the absolute configuration as depicted. It is the acetate derivative of prepacifenol which has been previously isolated from *Laurencia filiformis* collected in the USA.[86]

Sesquiterpene (3.3.2), 2,10-dibromo-3-chloro-7-chamigrene, which was isolated from both *A. parvula* and *L. filiformis* (in 0.010 and 0.036% yield base on the sea hare and

the red seaweed dry weight, respectively), had been previously found in a *Laurencia* seaweed by Howard and Fenical.[77]

Deoxyprepacifenol (3.2.1.41) was isolated from both *A. parvula* and *L. filiformis*. It was isolated in 0.042 and 0.135% yield from *A. parvula* and *L. filiformis* (base on the sea hare and the red seaweed dry weight, respectively). Deoxyprepacifenol had been previously isolated from a red seaweed *Laurencia* species.[78]

Pacifenol (3.2.1.45) was found in both *A. parvula* and *L. filiformis*. Its presence in the sea hare was determined by GCMS of a silica gel fraction of the crude extract while it was isolated in 0.143% yield (base on the seaweed dry weight) from *L. filiformis*. Pacifenol[79,80] had been previously isolated from a red seaweed *Laurencia* species.

In this present study the fimbrolide (3.3.3) was found only from the sea hare *A. parvula* as shown by a GCMS content of a flash Si gel fraction. It was first isolated from the red seaweed *Delisea fimbriata*[81] (a synonym for *D. pulchra*). It was obtained from *D. elegans*[82] and *D. pulchra*.[83,84] Moreover, it has been shown that the sea hares (*A. parvula* and *A. dactyloma*) obtained (3.3.3) and other compounds from their diet of *D. pulchra*.[87,88] Since several *Delisea* species including *D. pulchra* and *D. elegans* occur commonly in Tasmania, it seems likely that *A. parvula* in this study obtained (3.3.3) from its diet. However, no *Delisea* seaweeds could be found at the time and place of collection of *A. parvula*. The function of acquired algal secondary metabolites in sea hares remains a subject of debate.[90]

The purple pigment, the possible revised aplysioviolin (3.3.4) in this study was isolated only from *A. parvula*. The sea hare *Aplysia* biosynthesises aplysioviolin from r-phycoerythrin which is a red algal photosynthetic pigment.[89,90] A study on the purple pigment failed to established its function.[91]

Pentadecanal (3.3.10) and its aldol condensation product, namely (*E*)-2-tridecyl-2-heptadecenal (3.3.11) were obtained as minor components (0.005 and 0.010% yield base on the alga dry weight, respectively) only from *L. filiformis*. Although (*E*)-2-tridecyl-2-heptadecenal (3.3.11)[87] has been previously isolated from *Laurencia undulata* and *Laurencia papillosa* and its structure confirmed by synthesis from pentadecanal, this appears to be the first time that pentadecanal itself has been isolated from a *Laurencia* seaweed. The absence of pentadecanal and (*E*)-2-tridecyl-2-heptadecenal in *A. parvula* is hardly surprising since it is likely that they are utilised as part of the normal fatty acid catabolic processes of the animal.

The brine shrimp (*Artemia salina) bioassay*[92] was performed on the halogenated sesquiterpenes (3.3.1-3.3.2, 3.2.1.41, 3.2.1.45) and the purple pigment (3.3.4). The strongest activity of a ninety percent mortality was observed on deoxyprepacifenol (3.2.1.41) at a concentration of 230 μg/mL in sea water after 24 hours. The other compounds showed moderate activity (Table 3.5.5.1).

3.5 Experimental.

3.5.1 Collection.

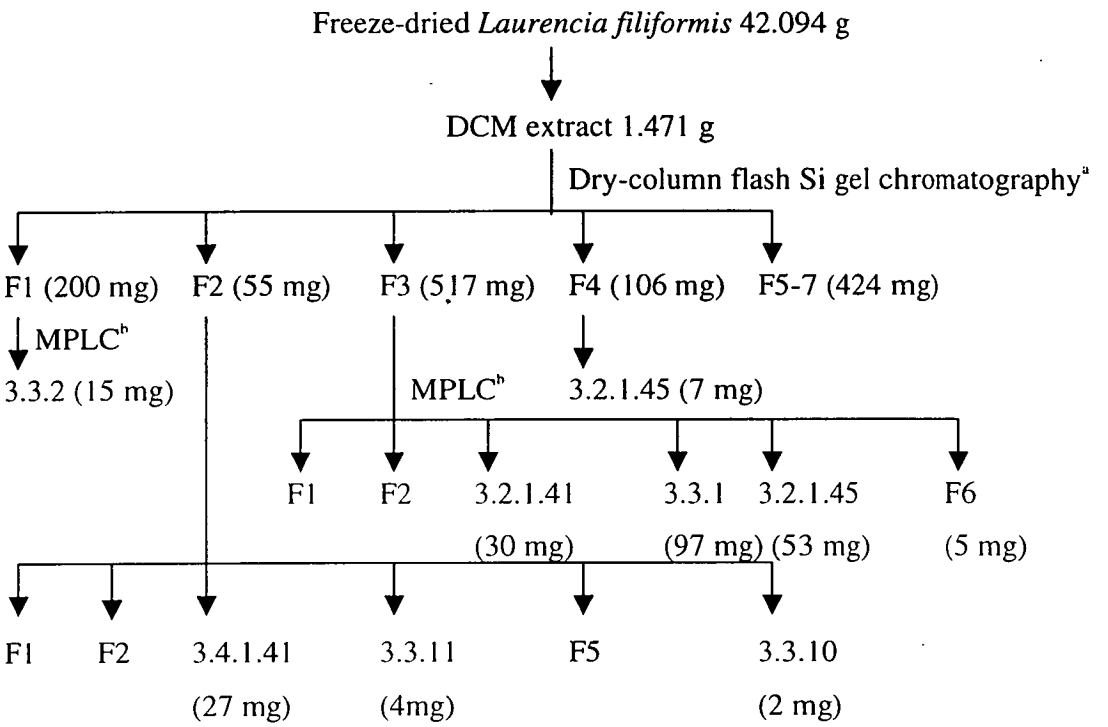
The sea hare *Aplysia parvula* and the red seaweed *Laurencia filiformis* were collected by scuba diving at a dept of 6 m at Taroona Beach, Hobart, Tasmania (42° 57' S, 147° 22' E) on 19th Feb 1999 and 10th Aug 1999, respectively. The approximate number of the sea hares was about 50 animals and the size of the sea hares was around 50-100 mm. Voucher specimens of *Aplysia parvula* have been lodged with the Tasmanian Museum & Art Gallery (reference number TMAGE 23224). Specimens of *Laurencia filiformis* have been deposited at the Tasmanian State Herbarium (reference number HO 504109).

3.5.2 Extraction procedure.

The red seaweed was frozen and freeze-dried. The dried sample (42.094 g) was exhaustively extracted with dichloromethane. The dichloromethane extract was concentrated on a rotary evaporator at a temperature below 30 °C to give a dark brown viscous tar (1.471 g).

Approximately 50 sea hare specimens were frozen and freeze-dried. The dried specimens (135 g) were extracted with petroleum ether, dichloromethane and methanol to give 3.032, 2.283 and 15.525 g of extracts, respectively.

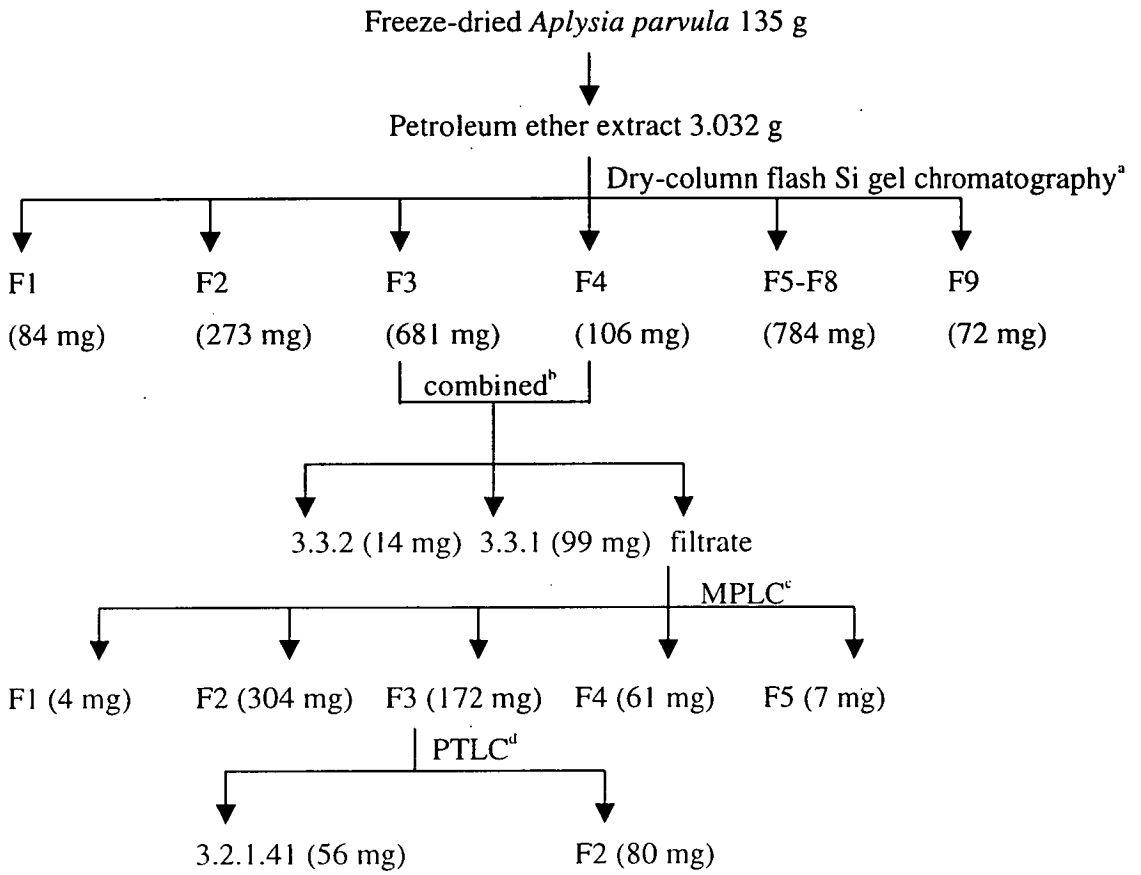
3.5.3 Separation procedure.



[a]using petroleum ether with increasing proportions of EtOAc as eluent.

[b]petroleum ether → 5-10% EtOAc/petroleum ether to give several nonpolar known compounds as well as the compound (3.3.2).

Scheme 3.5.3.1 Separation procedure used for the red seaweed *Laurencia filiformis*.



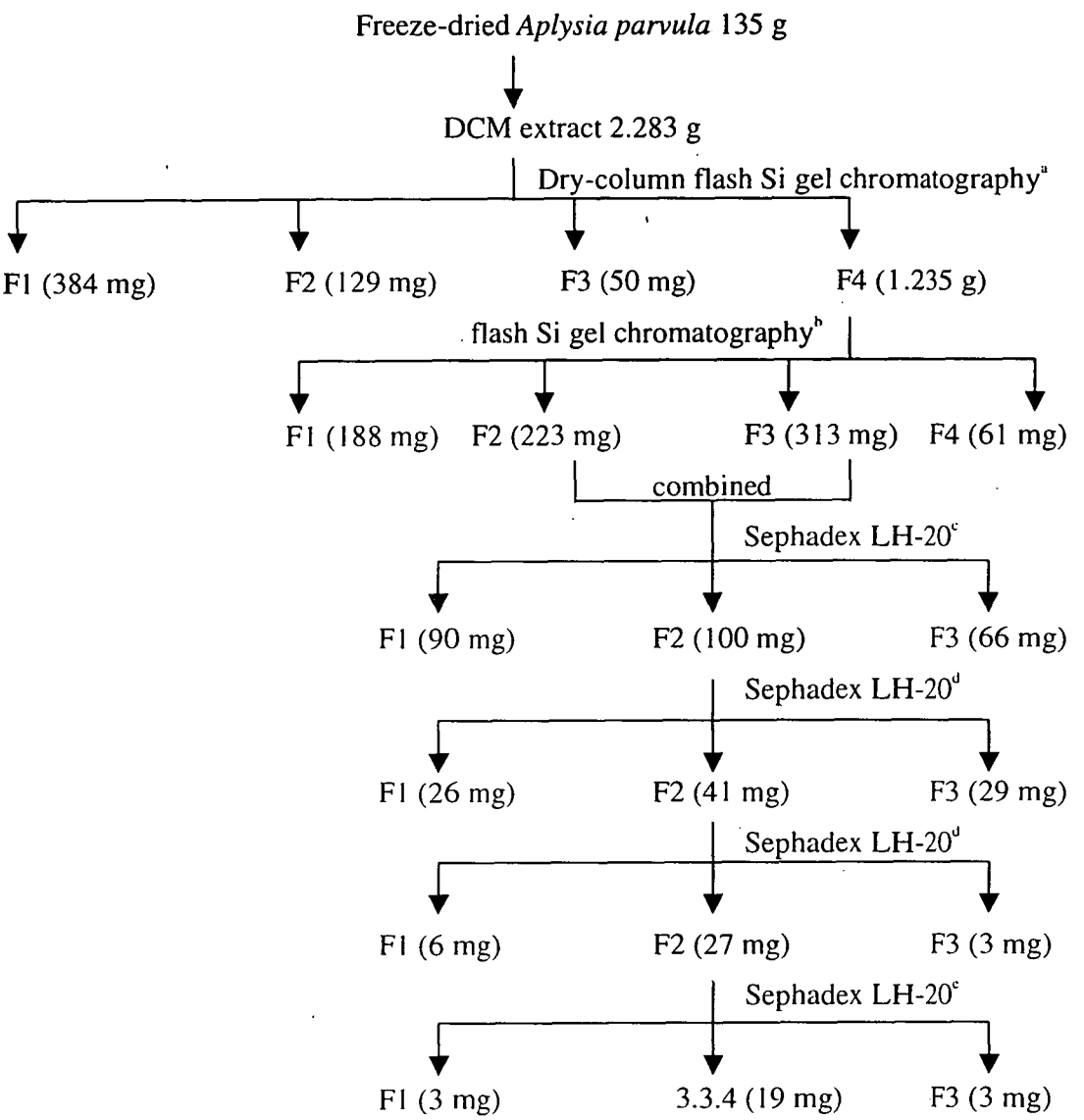
[a]using petroleum ether with increasing proportions of EtOAc as eluent.

[b]compound (3.3.2) was separated from F3 and the rest were combined with the filtrate after recrystallization with petroleum ether to give compound (3.3.1) from F4.

[c]petroleum ether → 5-10% EtOAc/petroleum ether.

[d]5% EtOAc/petroleum ether.

Scheme 3.5.3.2 Separation procedure used for petroleum ether extract of the sea hare *Aplysia parvula*.



[a]using dichloromethane, ethyl acetate, and methanol gradually increasing polarity [b]25% EtOAc/petroleum ether → 100% MeOH, [c]DCM→ MeOH, [d]MeOH, and [c]50%DCM/MeOH.

Scheme 3.5.3.3 Separation procedure used for dichloromethane extract of the sea hare *Aplysia parvula.*

3.5.4 Characterization of halogenated sesquiterpenes (3.3.1, 3.3.2, 3.2.1.41, 3.2.1.45), the fimbrolide (3.3.3), aplysioviolin (3.3.4), pentadecanal (3.3.10), and (*E*)-2-Tridecyl-2-heptadec-2-enal (3.3.11).

5-Acetoxy-2,10-dibromo-3-chloro-7,8-epoxy-α-chamigrene (3.3.1) was isolated as white needle crystals, m.p. 178.0-178.5 ^{0}C (petroleum ether), $[\alpha]_D$+62.5^0 (*c* 0.096, CH_2Cl_2), UV (CH_2Cl_2) λ_{max} (log ε) 205 (2.90) nm, IR (KBr) ν_{max} 1670, 1110, 940 cm^{-1}, ^{1}H and ^{13}C NMR data, see Table 3.3.1.1, HRLSIMS (Found: M+1, 468.98145 for $C_{17}H_{24}{}^{79}Br_2{}^{35}ClO_3$ requires M+1, 468.97807), EIMS m/z 468 (M$^+$, 0.1), 470 (0.2), 472 (0.1), 474 (0.05), 425 (0.5), 427 (1.2), 429 (0.8), 431 (1.8), 383 (1.4), 385 (3.1), 387 (2.1), 389 (0.8), 329 (4.4), 331 (6.6), 333 (2.1), 251 (6.7), 253 (7.5), 255(1.8), 187 (4.6), 189 (4.0), 176 (29.9), 178 (28.8), 147 (4.7), 149 (3.8), 119 (8.0), 97 (21.8), 91 (17.7), 53 (14.8), 43 (100).

2,10-Dibromo-3-chloro-7-chamigrene (3.3.2)[75] was obtained as a pale yellow oil, $[\alpha]_D$-38.5^0 (*c* 0.0005, CH_2Cl_2), ^{1}H and ^{13}C NMR data (this assigned ^{13}C NMR has not previously appeared in the literature), see Table 3.3.1.1, EIMS: m/z 396 (M$^+$, 0.3), 398 (0.8), 400 (0.4), 402 (0.1), 317 (2.7), 319 (3.3), 321 (8.6), 303 (9.9), 281 (3.8), 264 (6.7), 237 (14.4), 216 (3.2), 201 (28.1), 185 (17.0), 171 (11.8), 159 (23.3), 145 (46.4), 133 (100), 119 (50.1), 105 (48.5), 91 (75.7), 77 (51.2), 53 (41.9), 41 (67.3).

Deoxyprepacifenol (3.2.1.41)[78] was obtained as a white solid. The ^{1}H and ^{13}C NMR were consistent with the literature (see Table 3.3.3.1). The mass spectrum showed m/z 410 (1.7), 412 (4.1), 414 (3.0), 416 (0.7), 396 (0.3), 367 (2.1), 369 (5.3), 371 (3.6), 333 (2.8), 335 (0.8), 337 (0.5), 315 (0.7), 289 (2.1), 277 (0.7), 251 (3.4), 253 (3.1), 237 (0.6), 215 (4.8), 199 (3.5), 176 (23.7), 171 (10.3), 145 (6.9), 123 (71.6), 119 (37.5), 105 (21.3), 97 (67.6), 77 (45.4), 53 (40.5), 43 (100), 41 (55.3).

Pacifenol (3.2.1.45)[77,78] was obtained as a white solid. The ^{1}H and ^{13}C NMR were consistent with the literature (see Table 3.3.4.1). The mass spectrum showed m/z 408 (M$^+$-H_2O, 1.3), 410 (2.4), 412 (1.7), 372 (1.5), 374 (1.9), 376 (0.9), 329 (43.7), 331 (59.4), 333 (14.8), 213 (8.1), 215 (8.2), 199 (10.4), 171 (11.9), 133 (21.7), 119 (16.9), 91 (25.6), 43 (100).

4-Bromo-3-butyl-5-(dibromomethylene)-2(5*H*)-furanone (3.3.3)[81,82,83,84] was obtained as an oil. EI mass spectra showed m/z 386 (M$^+$, 7.8), 388 (24.1), 390 (24.0), 392 (8.6), 357 (1.7), 359 (6.0), 361 (6.0), 363 (1.7), 344 (15.5), 346 (46.6), 348 (43.1), 350

(14.7), 330 (1.4), 332 (3.9), 334 (3.7), 336 (1.3), 307 (49.1), 309 (100), 311 (49.1), 287 (1.7), 289 (5.7), 291 (5.9), 293 (1.7), 265 (22.8), 267 (44.8), 269 (21.6), 237 (4.5), 239 (8.6), 241 (4.5), 227 (7.8), 229 (8.6), 105 (19.8), 91 (34.5), 81 (35.3), 41 (47.4). The mass spectrum (Figure 3.3.5.1) was consistent with the literature.[79]

Aplysioviolin (3.3.4) was obtained as a purple solid film, dec. > 310 ^{0}C, $[\alpha]_D$-400 ^{0} (*c* 0.02, CH_2Cl_2), and $[\alpha]_D$-411 ^{0} (*c* 0.068, CH_3OH), UV (CH_2Cl_2) λ_{max} (log ε) 533 (3.76) nm and UV (CH_3OH) λ_{max} (log ε) 526 (4.00) nm, ^{1}H and ^{13}C NMR, see Table 3.3.6.1, HRLSIMS: (Found: M+1, 601.30080 for $C_{34}H_{41}N_4O_6$ requires M+1, 601.30260), ESI LCMS: 601 (MH^+), ESI MS/MS of (3.3.4): 601, 478, 297, 237, 222, 194, 167,

The dimethyl ester of compound (3.3.4): ESI MS/MS of the dimethyl ester of (3.3.4) (using diazomethane): 615, 492, 299, 257 and another way of 615, 492, 311, 237. EI MS: 600, 464, 431, 393, 331, 302, 213, 180, 131, 94, 69, 44, EI MS of the dimethyl ester of aplysioviolin: 614, 493, 478, 406, 374, 346, 302, 229, 149, 83, 43.

Pentadecanal (3.3.10) [86] revealed m/z 226 (M^+, 0.4), 208 (0.7), 198 (0.4), 193 (0.2), 182 (1.8), 165 (0.7), 152 (1.8), 138 (3.2), 124 (5.3), 110 (9.6), 96 (14.4), 82 (56.1), 67 (36.8), 57 (82.4), 55 (63.8), 43 (100), 41 (98.2). The structure was consistent with the MS literature data.[84] The ^{1}H NMR spectrum of the isolated pentadecanal (3.3.10) showed δ 0.88 (CH_3, t, *J*=2.7 Hz), 1.26 ((CH_2)$_9$(CH_2)$_3$$CH_2$CHO, s), 1.57 ($CH_2$)$_3$$CH_2$CHO, s), 2.42 ($CH_2$CHO, dt, *J*=1.9, 5.4 Hz), 9.77 (CHO, t, *J*=1.9 Hz).

(*E*)-2-Tridecyl-2-heptadec-2-enal (3.3.11) [87] showed m/z 434 (M^+, 44.7), 416 (0.6), 402 (1.5), 377 (0.2), 349 (0.2), 321 (0.6), 293 (2.3), 265 (7.6), 251 (11.2), 237 (13.6), 193 (2.3), 169 (1.5), 149 (3.0), 135 (7.6), 98 (29.5), 83 (34.8), 69 (32.6), 57 (50.0), 55 (55.8), 43 (100), 41 (56.8). The ^{1}H NMR spectrum showed δ 0.88 (2 -CH_3, t, *J*=6.3 Hz), 1.26 and 1.56 (CH_2)$_{12}$$CH_2CH_3$ and (CH_2)$_{11}$$CH_2CH_3$, s), 2.0 and 2.4 m, 2 -$CH_2CH_3$, s), 6.44 (CHCHO, t, *J*=7.6 Hz), 9.36 (CHO, s).The structure was consistent with the ^{1}H NMR literature data.[87]

3.5.5 Bioassay.

Brine shrimp (*Artemia salina*) bioassay[92] was performed on the halogenated sesquiterpenes (3.3.1, 3.3.2, 3.2.1.41, 3.2.1.45) and aplysioviolin (3.3.4). The strongest activity of ninety percent mortality was observed on deoxyprepacifenol (3.2.1.41) at concentration of 230 μg/mL in sea water after twenty four hours. The other compounds showed moderate activity (Table 3.5.5.1).

Table 3.5.5.1 Brine shrimp bioassay results of the sesquiterpenes and aplysioviolin.

compound	conc. (μg/mL)	percent deaths at 24 hrs[a]
control	-	-
(3.3.1)	220	44.4
(3.3.1)	22	55.6
(3.3.1)	2.2	77.8
(3.3.2)	120	33.3
(3.3.2)	12	49.1
(3.3.2)	1.2	81.5
(3.3.4)	180	51.8
(3.3.4)	18	74.1
(3.3.4.1)	1.8	63.0
(3.2.1.41)	230	90.0
(3.2.1.41)	23	51.9
(3.2.1.41)	2.3	51.9
(3.2.1.45)	210	66.7
(3.2.1.45)	21	40.7
(3.2.1.45)	2.1	44.4

[a]average of 5 replicates.

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Chapter 4. Ascidian *Polyandrocarpa lapidosa*.

4.1 General introduction and secondary metabolites from ascidians

Ascidians (syn. tunicates, sea squirts) are marine invertebrates which belong to the phylum Chordata, subphylum Urochordata or Tunicata and class Ascidiacea.[1] From a trend of chemistry of marine natural products, tunicates are increasing in the number of studies, and are second only to the field of microorganisms.[2] Kelecom indicated that during the period of 1977-1987, more than two thousand (2,122) metabolites had been isolated in total from the marine environment and ninety-eight of them (4.6%) were isolated from tunicates. These tunicates afforded metabolites biosynthesised by the mevalonate and nitrogen-containing pathways in 11.2% and 88.8%, respectively. Since this reflects the biological activities, the probability to isolate anticancer agents is the highest for tunicates and bryozoan. Antiviral drugs are widely distributed and the most active ones have been isolated from tunicates and sponges. Therefore, ascidians are still of interest to marine natural product research.

In 1989 Smith[3] produced a mini-review of the vanadium biochemistry in ascidians. Some organisms such as ascidians possess a variety of mechanisms for assimilating the particular transition metals needed for normal metabolic activity. Ascidians display a remarkable ability to sequester and reduce vanadium in specialised blood cells termed vanadocytes; similarly, iron-accumulating species possess ferrocytes.

Similarly, Rehder[4] reported on the structure and function of vanadium compounds in living organisms including ascidians in 1992. Binary complexes between vanadate and peptides (proteins) may be formed by hydrogen bonding or direct coordination. Several species among the ascidians accumulate vanadium to 10^7 times the level in sea water. The vanadium is concentrated up to 0.15 M in specialised blood cells (vanadocytes). Its function, which is thought to be an oxygen carrier, is still illusive.

In 1993 Bernard *et al.* reported some bioactive nitrogenous metabolites from ascidians.[5] Davidson also reviewed amino acid derived secondary metabolites from ascidians as producers of including peptides, polycyclic aromatic alkaloids, tryptophan-, lysine-, tyrosine- and phenylalanine-derived metabolites.[6]

Since that time several reviews of metabolites from ascidians have appeared. A review of bioactive metabolites from marine invertebrates was published by Bhakuni in 1994.[7] Two years later Kashman *et al.* reviewed some bioactive secondary metabolites: the latrunculins from sponges and pyrido[2,3,4kl]acridines from ascidians.[8]

In the same year Sings *et al.* reported on metabolites that were produced by symbiosis between tunicates of the family Didemnidae and two genera of blue-green algae *Synechocystis* and *Prochloron*.[9] In addition, a review of antifouling metabolites from ascidians was published by Davis *et al.*[10]

Recently Kobayashi *et al.*[11] presented a review of bioactive secondary metabolites from Okinawan sponges and tunicates which was divided into five sections—

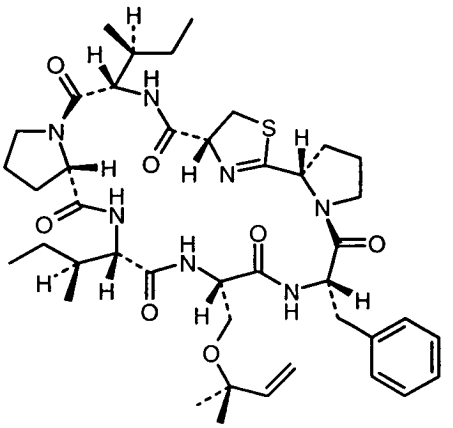
1) pseudodistomins and penararesidins
2) shimofuridins
3) manzamenones and *Plakortis* metabolites
4) taurospongin A and acetylene acids
5) theonezolides

This review will cover typical secondary metabolites reported from ascidians during the period 1994 to 2000 using SciFinder Scholar. Methods of isolation, structure elucidation and biological activity will be the focus where possible. All structures will be drawn the same as in the original publications wherever possible.

4.1.1 Cyclic peptide and macrocyclic alkaloids.

Carroll *et al.*[12] isolated a new cytotoxic cyclic heptapeptide, mollamide (4.1.1.1) from the ascidian *Didemnum molle*, which was collected by scuba diving at Net Reef which is located in the central part of the Great Barrier Reef, Australia. A methanol-dichloromethane crude extract was purified twice by silica gel column chromatography and finally on reverse phase HPLC with 65% acetonitrile in water to afford mollamide (4.1.1.1). X-ray crystallographic data indicated the stereochemistry of mollamide, while hydrolysis of mollamide and characterization of amino acids by derivatization with Marfey's reagent gave the absolute stereochemistry of mollamide as shown. All amino acids had the *L*-configuration. Mollamide showed cytotoxic activity against P388 murine

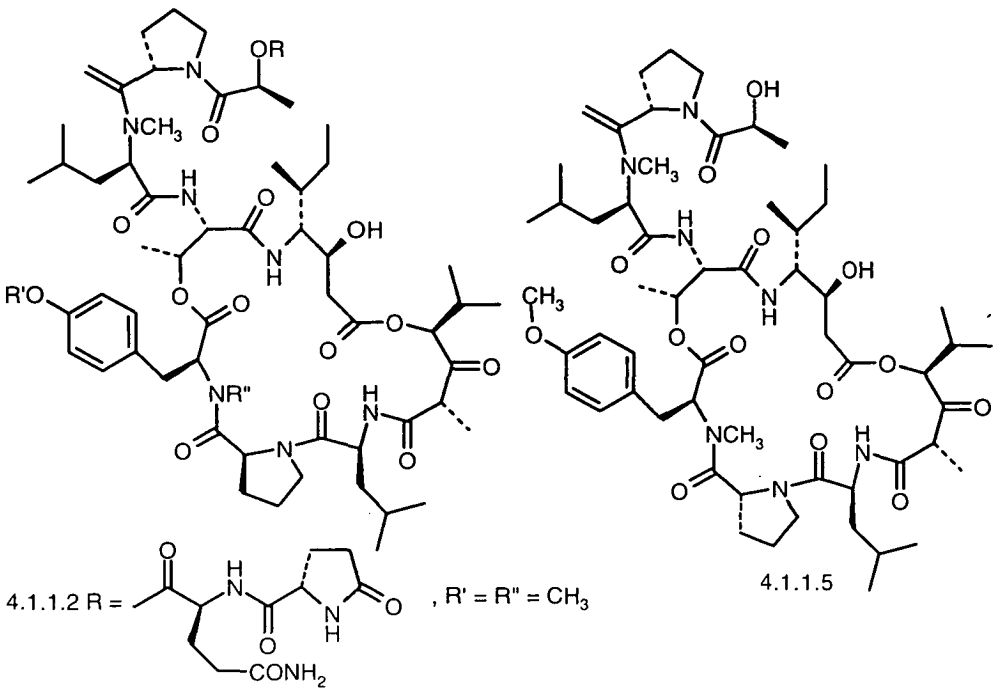
leukemia, A549 human lung carcinoma, HT29 human colon carcinoma, and CV1 monkey kidney fibroblast cells, as well as inhibiting RNA synthesis.



4.1.1.1

A new cyclodepsipeptide, didemnin H (4.1.1.2) was isolated from the ascidian *Tridemnum cyanophorum* (Didemnidae) by Boulanger *et al.*[13] The structure of didemnin H was determined by mass spectrometry, together with one- and two-dimensional NMR experiments.

In 1995 Abou-Mansour *et al.*[14] isolated the known didemnin B (4.1.1.3) and two new macrocycles namely [Tyr^5]didemnin B (4.1.1.4) and [D-Pro^4]didemnin B (4.1.1.5) from the ascidian *Trididemnum cyanophorum* (Didemnidae), collected by scuba diving off the coast of Guadeloupe Island, France. The structures were determined by FABMS and NMR spectroscopy while Marfey's analysis of the acid hydrolysates was used to determine their absolute configurations. The didemnins were tested for cytotoxic activity against human lymphoblastic leukemia cell lines.



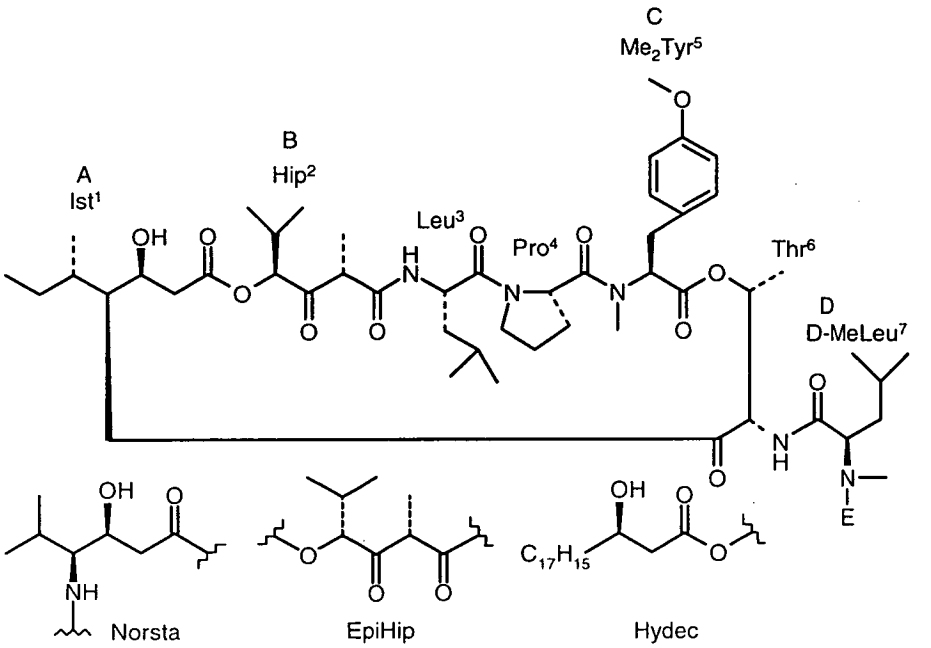
4.1.1.2 R = , R' = R" = CH_3

4.1.1.5

4.1.1.3 R = H, R' = R" = CH_3

4.1.1.4 R = R' = R" = H

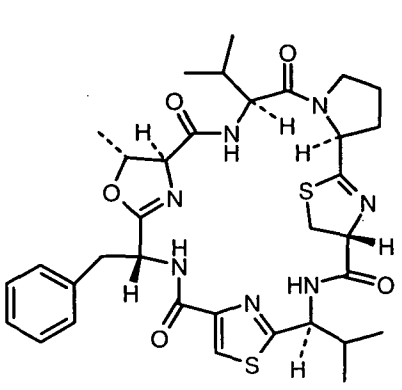
Seven new didemnins, namely didemnins M (4.1.1.6), N (4.1.1.7), X (4.1.1.8), and Y (4.1.1.9), nordidemnin N (4.1.1.10), epididemnin A_1 (4.1.1.11), as well as acyclodidemnin A (4.1.1.12) were isolated from the ascidian *Tridemnum solidum*, collected off the coast of St. George's Cay, Belize.[15] High-speed centrifugal countercurrent chromatography (HSCCC) was used to separate the metabolites. Each fraction containing didemnins was further purified by using a polystyrene-divinylbenzene copolymer gel, together with reversed phase and normal phase HPLC. Didemnins M, N, X, and Y showed cytotoxicity against P388 cells with IC_{50} of 2.0, 50, 2.0 and 2.0 ng/mL, respectively. While epididemnin A_1 and acyclodidemnin A showed much weaker cytotoxicity with IC_{50} of 2.0 and 0.2 μg/mL, respectively.



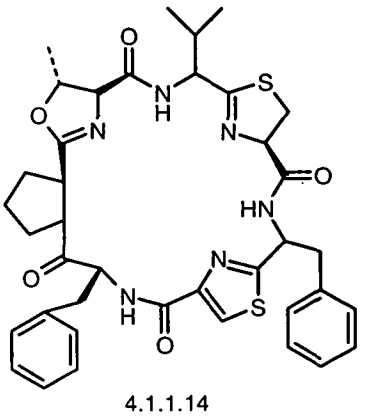
4.1.1.6 A=Ist, B=Hip, C=Me_2Tyr, D=D-MeLeu, E=-Pro-Lac-Gln-pGlu
4.1.1.7 A=Ist, B=Hip, C=Tyr, D=D-MeLeu, E=-Pro-Lac
4.1.1.8 A=Ist, B=Hip, C=Me_2Tyr, D=D-MeLeu, E=-Pro-Lac-Gln-Gln-Gln-Hydec
4.1.1.9 A=Ist, B=Hip, C=Me_2Tyr, D=D-MeLeu, E=-Pro-Lac-Gln-Gln-Gln-Gln-Hydec
4.1.1.10 A=Norsta, B=Hip, C=Tyr, D=D-MeLeu, E=-Pro-Lac
4.1.1.11 A=Ist, B=epiHip, C=Me_2Tyr, D=D-MeLeu, E=-H
4.1.1.12 A=Ist, B=Hip, C=Me_2TyrOH, D=MeLeu, E=-H

Toske *et al.*[16] reported a new cyclic heptapeptide, cyclodidemnamide (4.1.1.13) from the ascidian *Didemnum molle*, collected at the Cuyo Islands, in the Philippines. A 30% dichloromethane in methanol extract was partitioned between hexane and methanol. The methanol fraction was again partitioned between ethyl acetate (250 mL) and butanol (250 mL) in water. The structures were determined by HMQC, HMBC, COSY, ROESY and NOE spectroscopy. Absolute stereochemistries of the component amino acids were assigned using hydrolysis, derivatization and chiral GCMS analysis. Cyclodidemnamide showed weak cytotoxicity toward human colon tumour cells, HCT116 *in vitro* with an ED_{50} of 16 μg/mL.

The structure of lissoclinamide 4 (4.1.1.14) from the ascidian *Lissoclinum patella* was confirmed by synthesis.[17]



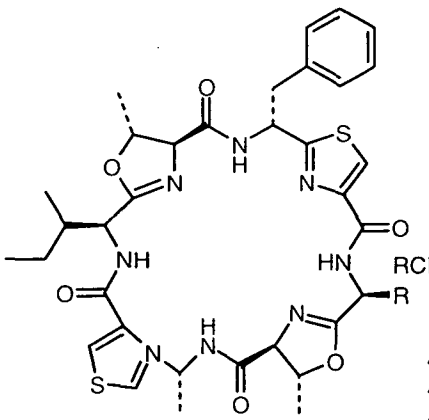
4.1.1.13



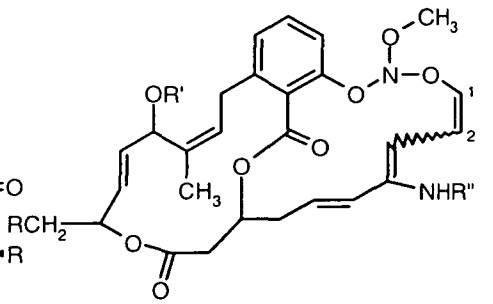
4.1.1.14

Ishida *et al.*[18] reported solution conformations of cyclic hexapeptides, petellamides B (4.1.1.15) and C (4.1.1.16) from the ascidian *Lissoclinum patella* by using NMR spectroscopy and molecular dynamics. The patellamides B and C studied in this publication were synthesised.

Seven macrocyclic alkaloids, aplidites A-G (4.1.1.17-4.1.1.23) were isolated from the ascidian *Aplidium* sp., collected from the Great Australian Bight by commercial trawling.[19] A methanol soluble fraction was purified using Sephadex LH-20 chromatography, reverse phase rapid silica filtration and Ultracarb ODS 20 HPLC. The structures were determined using spectroscopic analysis, derivatization and degradation.



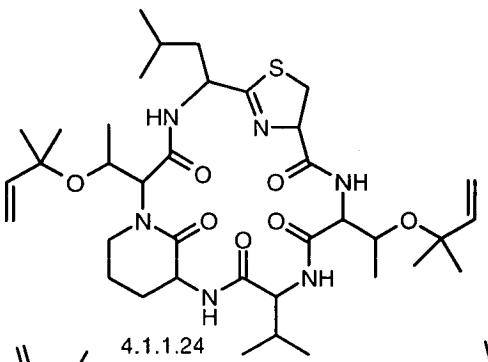
4.1.1.15 R=$CH_2CH(CH_3)_2$
4.1.1.16 R=$CH(CH_3)_2$



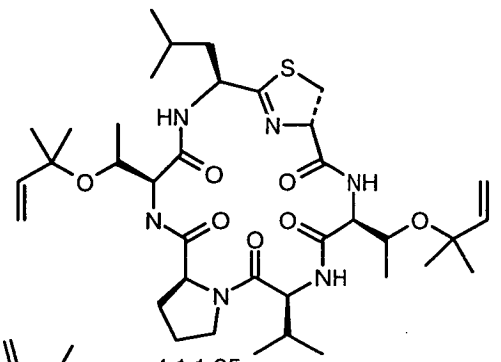
4.1.1.17 R=R'=R"=H, $\Delta^{1,2}$ *E* or *Z*
4.1.1.18 R=R'=R"=H, $\Delta^{1,2}$ Z or E
4.1.1.19 R=OH, R'=R"=H, $\Delta^{1,2}$ *E* or *Z*
4.1.1.20 R=OH, R'=R"=H, $\Delta^{1,2}$ *Z* or *E*
4.1.1.21 R=OH, R'=Ac, R"=H, $\Delta^{1,2}$ *E* or *Z*
4.1.1.22 R=OH, R'=Ac, R"=H, $\Delta^{1,2}$ *Z* or *E*
4.1.1.23 R=OAc, R'=R"=H, $\Delta^{1,2}$ *Z* or *E*

In 1996 patellins 1-6 (4.1.1.24-4.1.1.29), and trunkamide A (4.1.1.30) were isolated by Carroll *et al.*[20] from the colonial ascidian *Lissoclinum patella* and the ascidian *Lissoclinum* sp., collected from Viti Levu, Fiji and on the Great Barrier Reef, Australia,

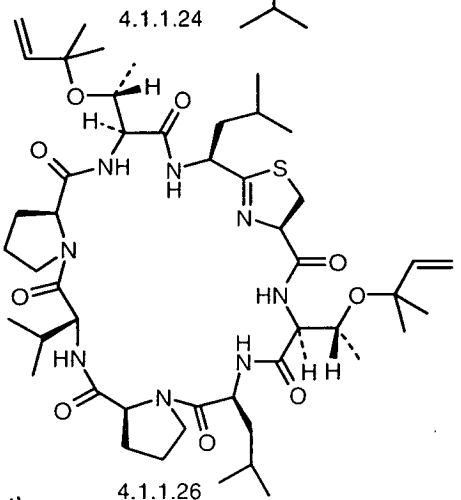
respectively. All structures were determined using NMR and mass spectroscopic methods analysis of hydrolyzed peptides and derivatization by Marfey's procedure.



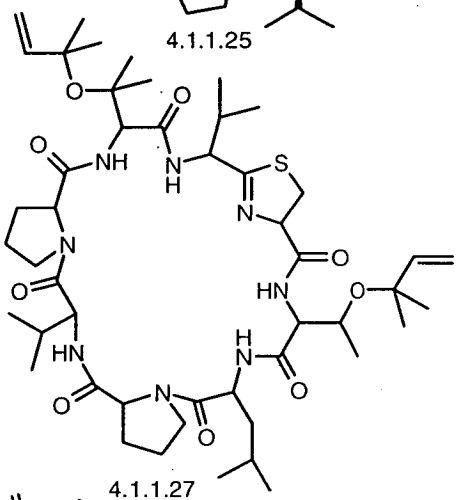
4.1.1.24



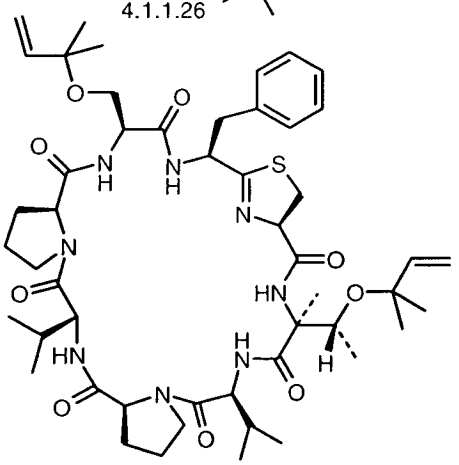
4.1.1.25



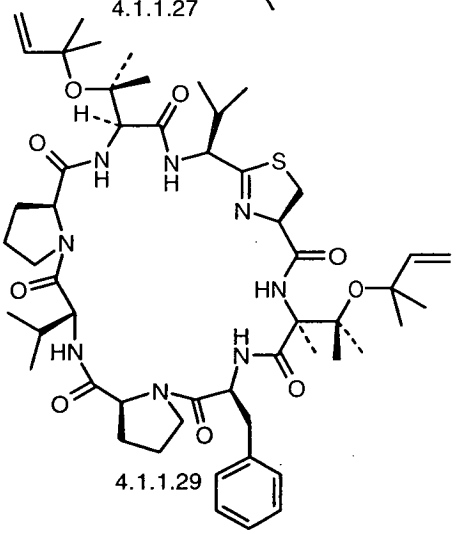
4.1.1.26



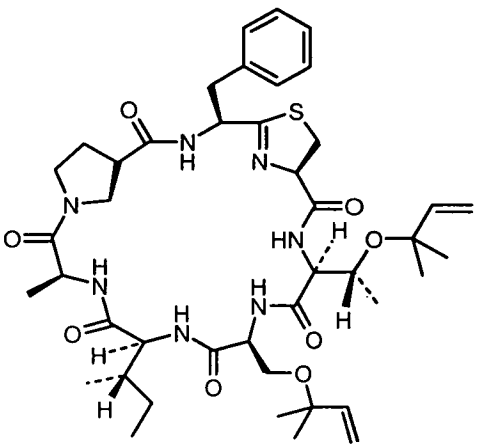
4.1.1.27



4.1.1.28

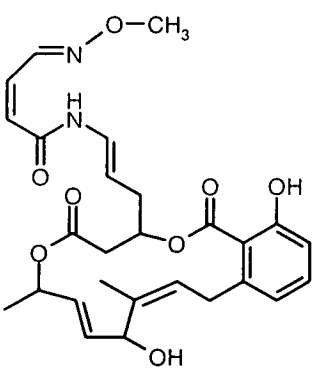


4.1.1.29

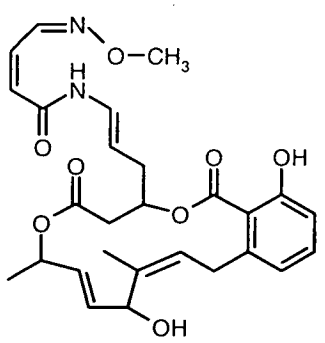


4.1.1.30

Galinis *et al.*[21] isolated two new macrolides, lobatamides A (4.1.1.31) and B (4.1.1.32) from the ascidian *Aplidium lobatum*, collected from the southwestern coast of Australia due west of Hillary Boat Harbour. The structures were determined by NMR experiments (COSY, difference NOE, HMQC, HMBC spectroscopy), and mass spectrometry (HRFABMS). A CIMS deuterium exchange experiment using ND_3 as the ionizing agent indicated the presence of three exchangeable protons. Exchanges were determined by comparing spectra obtained using ND_3 as the reagent gas with those from NH_3.



4.1.1.31

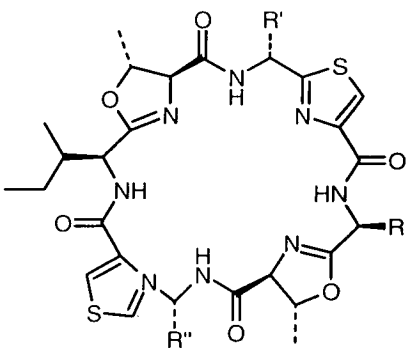


4.1.1.32

In 1998 the conformations of patellaides A (4.1.1.33), B (4.1.1.15), and E (4.1.1.34), which were isolated from the ascidian *Lissoclinum patella*, were studied by variable temperature circular dichroism in methanol. Studies of the binding properties of the lissoclinum cyclopeptides (4.1.1.15), (4.1.1.33-4.1.1.34) with zinc (II) and copper (II) ions, using circular dichroism (CD) and mathematical modelling techniques were described. CD provides a method of assigning conformation to members of the patellamide family. The CD spectrum of a typical polypeptide derives from the spectroscopic

interaction of an amide chromophore with its ordered neighbouring amides. A particular CD spectrum profile can be correlated with a particular oligopeptide conformation. The patellamides have a peptide-like core containing oxazoline and thiazole rings. The observed CD of the patellamides must originate from spectroscopic interactions between electronic excitations based on the heterocyclic rings, the thiazoles in particular. The study showed that CD was an important tool for determination of metal binding constants uncomplicated by paramagnetism or free metal contributions and providing insight into multi-binding characteristics.[22]

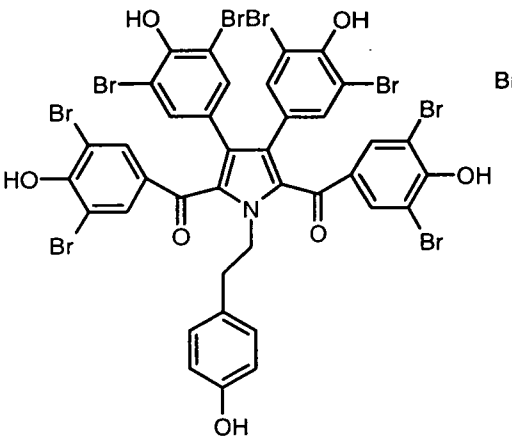
Wipf *et al.*[23] reported the total synthesis of a cycloheptapeptide alkaloid, trunkamide A (4.1.1.30), which was isolated from the colonial ascidian *Lissoclinum* sp. It was prepared via segment condensations and an oxazoline-thiazoline interconversion.



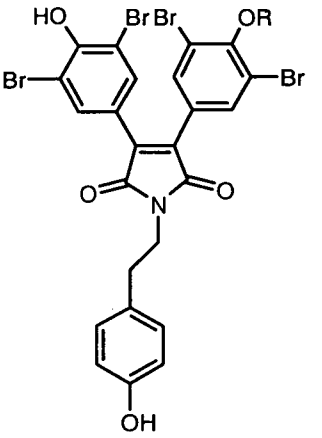
4.1.1.33 R=$CH(CH_3)CH_2(CH_3)$, R'=$CH(CH_3)_2$, R"=$CH(CH_3)_2$
4.1.1.34 R=$CH(CH_3)_2$, R'=CH_2Ph, R"=$CH(CH_3)_2$

4.1.2 Polyaromatic alkaloids.

Polycitone A (4.1.2.1), and polycitrins A (4.1.2.2) and B (4.1.2.3) were isolated from the ascidian *Polycitor* sp., which was collected at Sodwana Bay, South Africa. All structures were established on the basis of NMR spectroscopy and for polycitone A also by single crystal X-ray diffraction analysis.[24] In 1995 a total synthesis of polycitrin A (4.1.2.2) was described based on the formation of 3,4-bisarylpyrrole-2,5-dicarboxylic acids from 3-arylpyruvic acids by Terpin *et al.*[25]

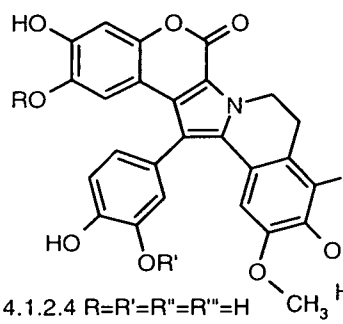


4.1.2.1

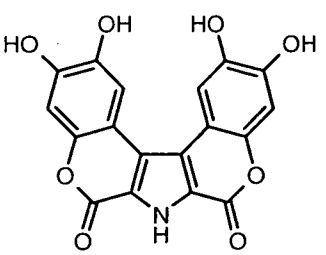


4.1.2.2 R=H
4.1.2.3 R=CH_3

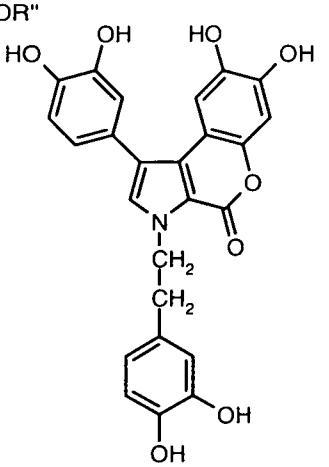
Urban *et al.*[26] isolated a new aromatic alkaloid, lamellarin S (4.1.2.4) and a known metabolite, lamellarin K (4.1.2.5) from the ascidian *Didemnum* sp. The specimen was collected from the coast off Durras, New South Wales, Australia. The crude ethanol extract of the ascidian, which was purple in colour, was partitioned into dichloromethane soluble fraction and dichloromethane insoluble fraction. The dichloromethane insoluble fraction was purified using Sephadex LH-20 chromatography and reverse phase HPLC to afford the metabolites. The purple ethanol crude extract of this ascidian showed slight growth inhibitory properties against a *Serratia* sp. and a *Micrococcus* sp.



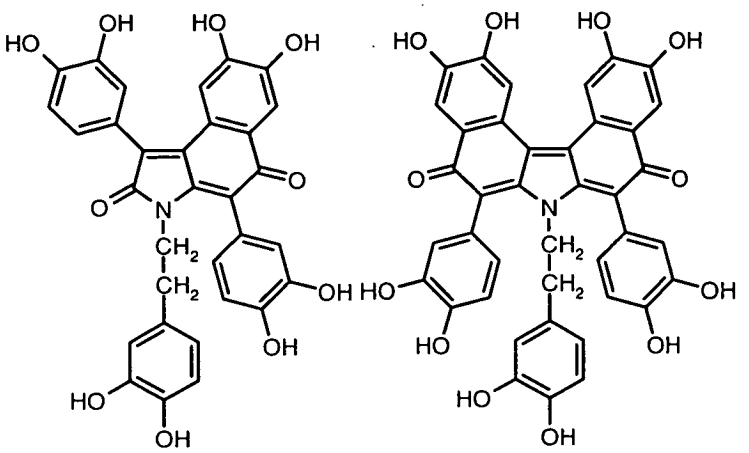
4.1.2.4 R=R'=R"=R'"=H
4.1.2.5 R=R'=R"=CH_3, R'"=OH



4.1.2.6

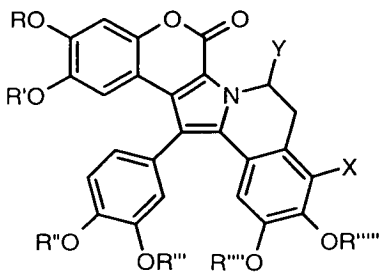


4.1.2.7

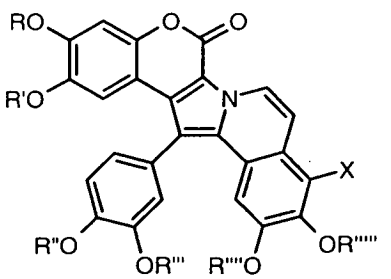


4.1.2.8 4.1.2.9

Four new aromatic alkaloids, ningalins A-D (4.1.2.6-4.1.2.9) were isolated from an unknown ascidian *Didemnum* sp. collected near Ningaloo Reef in Western Australia by Kang *et al.*[27] Davis *et al.* isolated a new alkaloid, lamellarin Z (4.1.2.10) and eight known compounds, lamellarin A (4.1.2.11), B (4.1.2.12), C (4.1.2.13), E (4.1.2.14), G (4.1.2.15), L (4.1.2.16), D triacetate (4.1.2.17), and lamellarin N triacetate (4.1.2.18) from a Great Barrier Reef ascidian *Didemnum chartaceum.*[28]



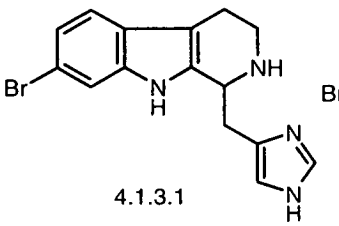
4.1.2.10 R=R''''=CH_3, R'=R''=R'''=R'''''=X=Y=H
4.1.2.11 R=R''=R'''=H, R'=R''''=R'''''=CH_3, X=OCH_3, Y=OH
4.1.2.13 R=R''=Y=H, R'=R'''=R''''=R'''''=CH_3, X=OCH_3
4.1.2.14 R=R'''=Y=H, R'=R''=R''''=R'''''=CH_3, X=OH
4.1.2.15 R=R''=R''''=CH_3, R'=R'''=R'''''=X=Y=H
4.1.2.16 R=R=R'''=R'''''=X=Y=H, R'=R''=R''''=CH_3



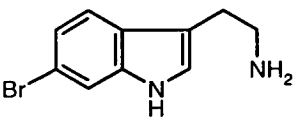
4.1.2.12 R=R''=H, R'=R'''=R''''=R'''''=CH_3, X=OCH_3
4.1.2.17 R=R''=R'''''=Ac, R'=R'''=R''''=CH_3, X=H
4.1.2.18 R=R'''=R'''''=Ac, R'=R''=R''''=CH_3, X=H

4.1.3 Carboline and indole derivative alkaloids.

Searle *et al.*[29] reported a new indole, lissoclin C (4.1.3.1) and a known compound, 6-bromotryptamine (4.1.3.2) from the ascidian *Lissoclinum* sp., which was collected from Michelmas Reef, Great Barrier Reef, Australia.

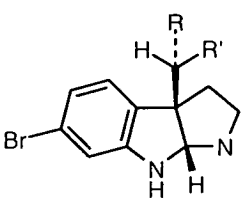


4.1.3.1

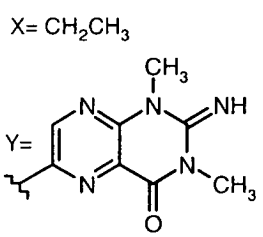


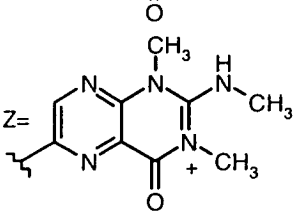
4.1.3.2

Two pteridine-containing bromophysostigmine alkaloids, urochordamine A (4.1.3.3) and B (4.1.3.4) were isolated from two ascidians *Ciona savignyi* (tunic only) and *Botrylloides* sp. (whole body). These compounds were observed to be larval metamorphosis promoters of the ascidian *Halocynthia roretzi* and induced metamorphosis of the pediveliger larvae of the mussel *Mytilus edulis galloprovincialis*. When left standing in protic solvents, urochordamine A and B were converted to more polar derivatives, urochordamines A' (4.1.3.5) and B' (4.1.3.6), respectively. Effects of urochordamines A, B, A' and B' on larval metamorphosis at a concentration of 25 μg/mL showed that the promoting activity of urochordamine B was lower than that of urochordamines A or A'. However, urochordamine B' showed no promoting activity at the concentration of 25 μg/mL. Urochordamine A' induced larval metamorphosis even at a concentration of 0.25 μg/mL, while urochordamine A was ineffective at a concentration of 2.5 μg/mL.[30]



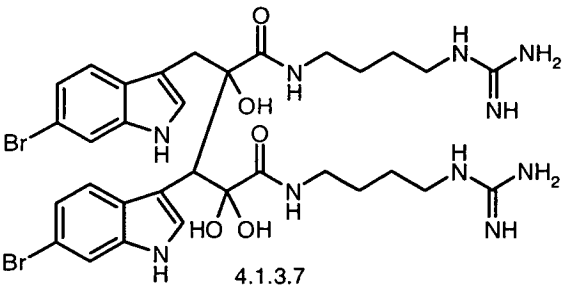
4.1.3.3 R=X, R'=Y
4.1.3.4 R=Y, R'=X
4.1.3.5 R=X, R'=Z
4.1.3.6 R=Z, R'=X





Swersey *et al.*[31] isolated a modified peptide dimer, eudynstyelamide (4.1.3.7) from the ascidian *Eusynstyela misakiensis*, which was collected at the northern and northeastern

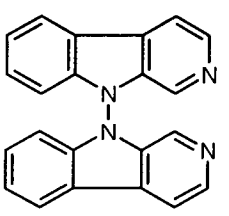
reefs of Siquijor Island, Philippines. The metabolite was nontoxic towards the human colon tumour cell line HCT116 at concentrations up to 100 μg/mL.



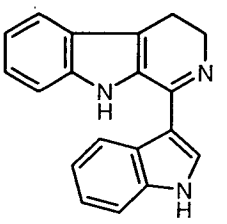
4.1.3.7

A β-carboline dimer (4.1.3.8) was isolated from an ascidian *Didemnum* sp., which was collected from Sykes Reef in the Capricorn Bunker Group of the southern Great Barrier Reef, Australia.[32]

In 1995 Massiot *et al.*[33] reported a correct structure, based on total synthesis, of isoeudistomin U (4.1.3.9), a natural product previously isolated from the ascidian *Lissoclinum fragile*. The synthetic and natural products were identical by comparing the ^{13}C NMR values between the synthetic data and the literature data.[34] This paper mentioned that the synthetic isoeudistomin U was inactive in a tubulin bioassay referring to the previous work done by Zavala *et al.*[35] in 1978.

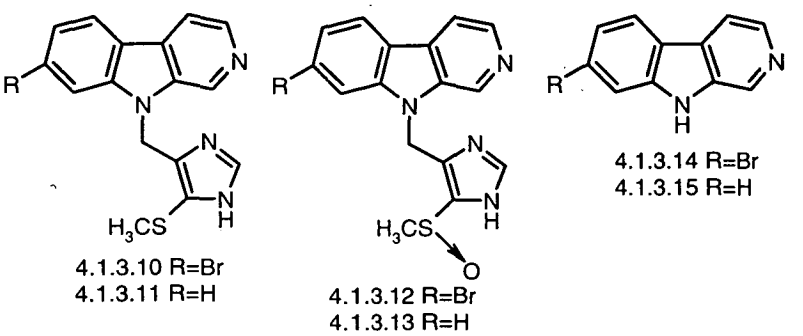


4.1.3.8



4.1.3.9

Four new N-9 substituted β-carbolines, didemnolines A-D (4.1.3.10-4.1.3.13) along with eudistomin O (4.1.3.14), the β-carboline (4.1.3.15), and 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (4.1.8.6) were reported from the ascidian *Didemnum* sp. by Schumacher *et al.*[36] The specimen was collected near the island of Rota, Northern Mariana Islands.



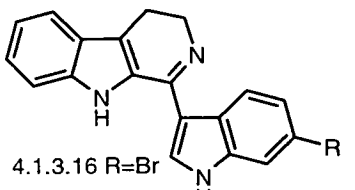
Kang *et al.*[37] isolated two new dihydro-β-carbolines, 19-bromoisoeudistomin U (4.1.3.16) and isoeudistomin U (4.1.3.9) from an undescribed Western Australian ascidian of the genus *Eudistoma* in 1996. In the same year *N,N*-didesmethylgrossularine-1 (4.1.3.17) was isolated from the ascidian *Polycarpa aurata* collected in Chuuk, Federated States of Micronesia by Abas *et al.*[38]

In 1997 four new alkaloids, didemnimides A-D (4.1.3.18-4.1.3.21) were isolated from the Caribbean mangrove ascidian *Didemnum conchyliatum* by Vervoort *et al.*[39] The ascidian was collected from the blades of the seagrass *Thalassia testudinum* in the mangrove channels of Sweetings Cay, near Grand Bahama Island, Bahamas. Didemnimide D was the most active deterrent of feeding of the carnivorous wrasse *Thalassoma bifasciatum.* In 1998 Vervoort *et al.*[40] reported chemical defense activity by fractions containing the indole-maleimide-imidazole alkaloids didemnimides A-D (4.1.3.18-4.1.3.21) from the Caribbean ascidian *Didemnum conchyliatum.*

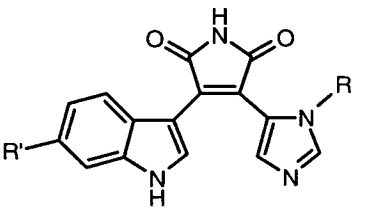
Berlinck *et al.*[41] found the aromatic alkaloids didemnimide A (4.1.3.18), D (4.1.3.21), E (4.1.3.22), granulatimide (4.1.3.23), and isogranulatimide (4.1.3.24) from the ascidian *Didemnum granulatum*, which was collected from Araca beach, Sao Sebastiao, Sao Paulo State, Southeastern Brazil. The structures of metabolites (4.1.3.18, 4.1.3.23-4.1.3.24) were also confirmed by synthesis. Granulatimide and isogranulatimide are G2 specific cell cycle checkpoint inhibitors and were identified through a rational screening program.

Vevoort *et al.*[42] isolated a new cyclized didemnimide alkaloid (4.1.3.24) and the known didemnimide A-D (4.1.3.18-4.1.3.21) from the Caribbean ascidian *Didemnum conchyliatum.* A new indole alkaloid (4.1.3.25) was isolated from the ascidian *Dendrodoa grossularia*, which was collected in Brittany during low tides (Ile Callot, France). A relative stereochemistry of (4.1.3.25) was assigned by Loukaci *et al.*[43] Franco *et al.*[44]

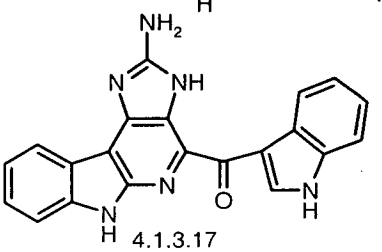
isolated five new indole alkaloids, meridianins A-E (4.1.3.26-4.1.3.30) from the ascidian *Aplidium meridianum*, which was collected by trawling near the South Georgia Islands. Meridianins B-E displayed cytotoxicity toward LMM3 (murine mamarian adenocarcinoma cell line).



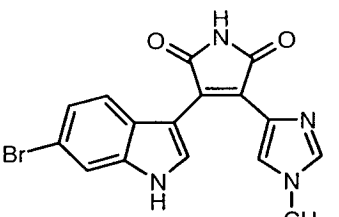
4.1.3.16 R=Br



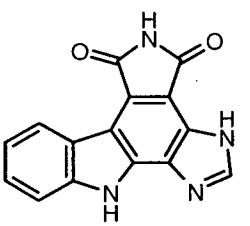
4.1.3.18 R=R'=H
4.1.3.19 R=H, R'=Br
4.1.3.20 R=CH_3, R'=H
4.1.3.21 R=CH_3, R'=Br



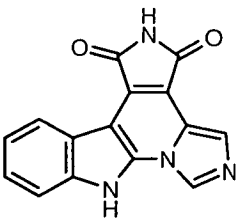
4.1.3.17



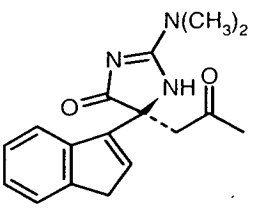
4.1.3.22



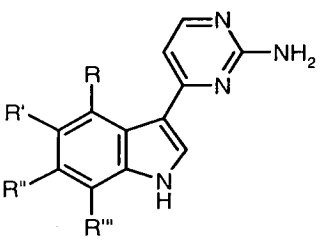
4.1.3.23



4.1.3.24

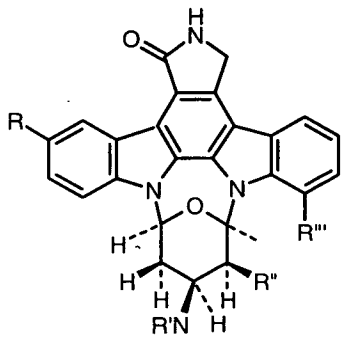


4.1.3.25



4.1.3.26 R=OH, R'=R"=R'"=H
4.1.3.27 R=OH, R'=R'"=H, R"=Br
4.1.3.28 R=R"=R'"=H, R'=Br
4.1.3.29 R=R'=R'"=H, R"=Br
4.1.3.30 R=OH, R'=R"=H, R'"=Br

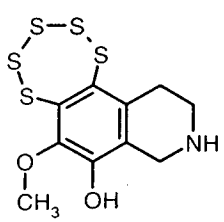
In 1999 two new indolocarbazole alkaloids, 3-hydroxy-3'-demethoxy-3'-hydroxystaurosporine (4.1.3.31) and 11-hydroxy-4'-N-demethylstaurosporine (4.1.3.32) were isolated from the ascidian *Eudistoma toealensis* and its predator, the flatworm *Pseudoceros* sp.[45] The specimens were collected in areas of Chuuk, Micronesia.



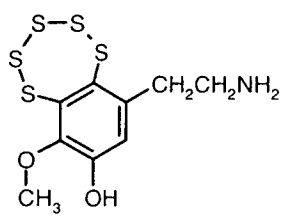
4.1.3.31 R=R"=OH, R'=CH_3, R'"=H
4.1.3.32 R=R'=H, R"=OCH_3, R'"=OH

4.1.4 Sulfur-containing alkaloids.

Litaudon *et al.*[46] isolated a new pentathiepin derivative, lissoclinotoxin B (4.1.4.1) from the ascidian *Lissoclinum perforatum* collected in Dinard Harbour, northern Brittany. They also obtained a correct structure of lissoclinotoxin A (4.1.4.2). The structures were determined using inverse long range heteronuclear correlations and mass spectrometric analyses.



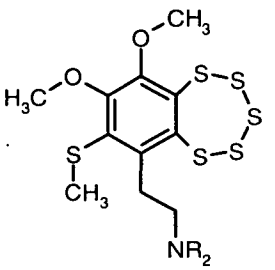
4.1.4.1



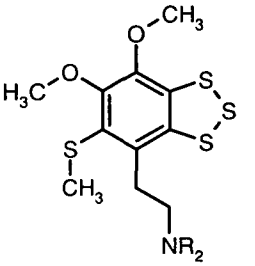
4.1.4.2

Pentathiepins, namely *N,N*-dimethyl-5-(methylthio)varacin (4.1.4.3) and trithianes, 3,4-dimethoxy-6-(2'-*N,N*-dimethylaminoethyl)-5-(methylthio)benzotrithiane (4.1.4.4) were isolated from the ascidian *Lissoclinum japonicum*, which was collected at Malakal Passage, Palau by Compagnone *et al.*[47] In addition, an inseparable 2:3 mixture of 5-(methylthio)-varacin (4.1.4.5) and trithiane (4.1.4.6) was obtained from *Lissoclinum* sp. which was collected at Ant Atoll, Pohnpei, Federated States of Micronesia. Similarly, 3,4-desmethyl-varacin (4.1.4.7) was isolated from *Eudistoma* sp., which was collected at Aru Pass, Pohnpei. Pentathiepin (4.1.4.3) and trithiane (4.1.4.4) showed mild antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* but displayed no selectivity in the National Cancer Institute's 60 cell-line panel. The mixture of (4.1.4.5) and (4.1.4.6) was the most active inhibitor of protein kinase C (IC_{50} of 0.3 μg/mL). Trithiane (4.1.4.4) was twice as active as the pentathiepin (4.1.4.3) but both of them were less active

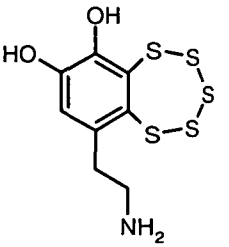
than the mixture. The activity of varacin and related compounds might be due to evolution of sulfur.



4.1.4.3 R=Me
4.1.4.5 R=H

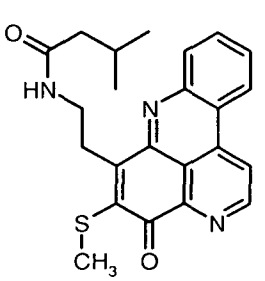


4.1.4.4 R=Me
4.1.4.6 R=H

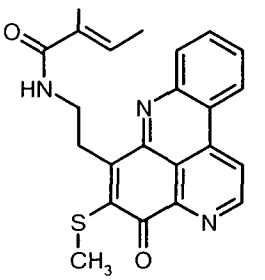


4.1.4.7

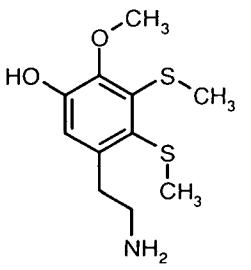
Searle *et al.*[29] isolated five new alkaloids, lissoclin A (4.1.4.8), lissoclin B (4.1.4.9), lissoclin C (4.1.3.1), lissoclinotoxin C (4.1.4.10), the dimeric lissoclinotoxin D (4.1.4.11) together with known compounds lissoclinotoxin A (4.1.4.2), and 6-bromotryptamine (4.1.3.2) from the ascidian *Lissoclinum* sp. The specimen was collected from the Great Barrier Reef, Australia.



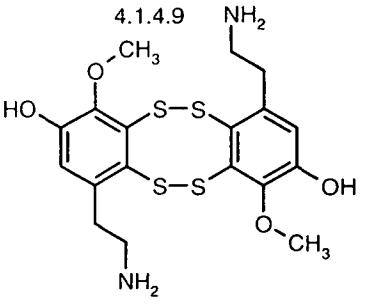
4.1.4.8



4.1.4.9

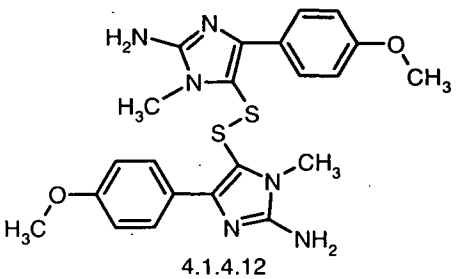


4.1.4.10



4.1.4.11

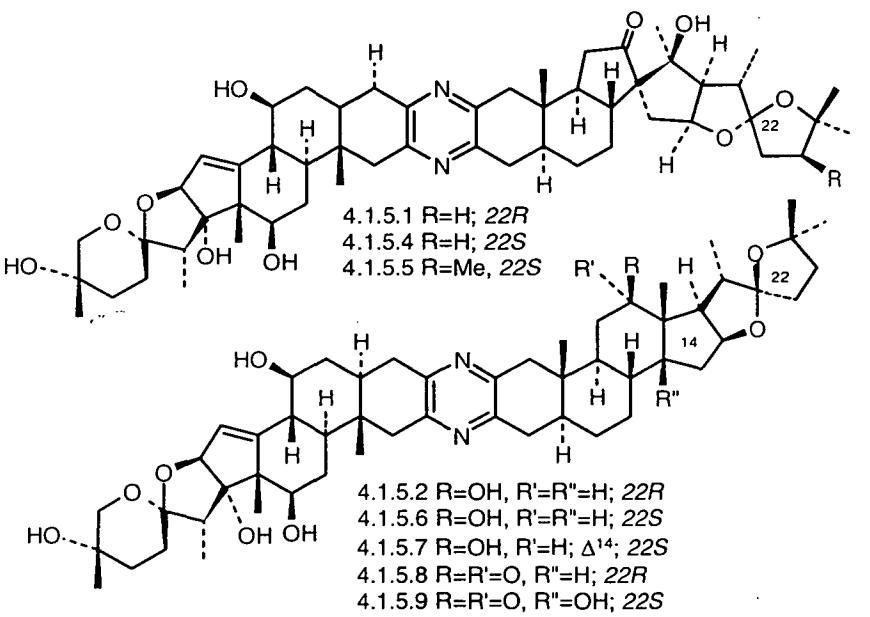
In 1996 two new alkaloids, polycarpine (4.1.4.12) and *N,N*-didesmethylgrossularine-1 (4.1.3.17) were isolated from the ascidian *Polycarpa aurata*, which was collected in Chuuk, Federated States of Micronesia.[33]



4.1.4.12

4.1.5 Steroidal alkaloids.

In 1994 Fukuzawa *et al.*[48] isolated a dimeric steroidal alkaloid, ritterazine A (4.1.5.1) from the ascidian *Ritterella tokioka* collected from the Izu Peninsula, southwest of Tokyo. A year later ritterazines B (4.1.5.2) and C (4.1.5.3) were also isolated from the same ascidian collected from the same location by the same group.[49] Ritterazine A, B and C exhibited cytotoxicity against P388 murine leukemia cells with IC_{50} values of 0.0038 μg/mL, 0.018 and 9.4 ng/mL, respectively.

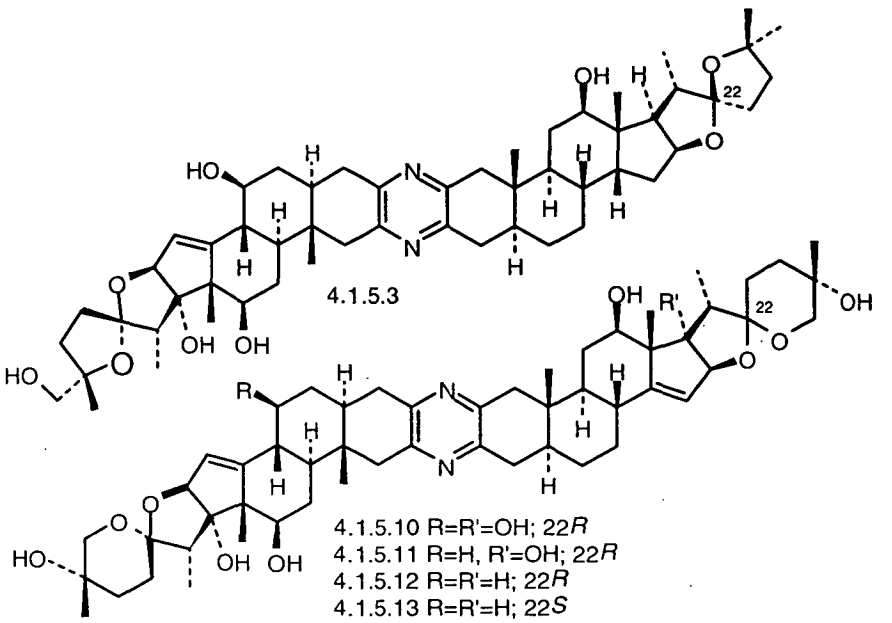


4.1.5.1 R=H; *22R*
4.1.5.4 R=H; *22S*
4.1.5.5 R=Me, *22S*

4.1.5.2 R=OH, R'=R"=H; *22R*
4.1.5.6 R=OH, R'=R"=H; *22S*
4.1.5.7 R=OH, R'=H; Δ^{14}; *22S*
4.1.5.8 R=R'=O, R"=H; *22R*
4.1.5.9 R=R'=O, R"=OH; *22S*

Ten more ritterazines D-M (4.1.5.4-4.1.5.13) were isolated from the ascidian *Ritterella tokioka* collected from the Izu Peninsula, southwest of Tokyo in 1994 by the same group.[50] Ritterazines D-M showed potent cytotoxicity against P388 murine leukemia cells with IC_{50} values of 16, 3.5, 0.73, 0.73, 16, 14, 13, 9.5, 10 and 15 ng/mL, respectively. In the same year Jeong *et al.*[51] reported a total synthesis of (+)-ritterazine K (4.1.5.11). In 1996 Fukuzawa *et al.*[52] also determined the orientation of two steroidal units in ritterazine A (4.1.5.1) using ^{15}N-HMBC spectroscopy. Since no ^{1}H-^{1}H or ^{1}H-^{13}C connectivities were

observed between the atoms of the two steroidal units, determination the orientation of the steroidal units with respect to the pyrazine ring was rather difficult.

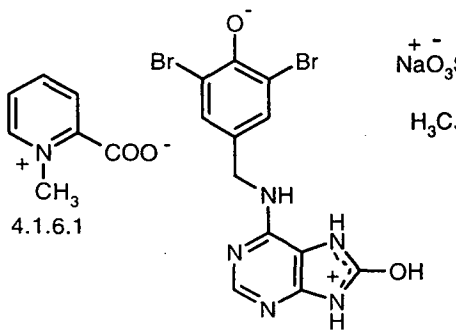


4.1.6 Zwitterionic alkaloids and nitrogen-containing salts.

In 1996 low molecular weight metabolites from three species of ascidians, *Aplidium nordmani*, *Styela partita* and *Botrillus leachii*, which were collected from the Lagoon of Venice, Italy, were reported.[53] Fractions containing homarine (4.1.6.1) were obtained by droplet counter current chromatography of *n*-butanol extracts of *Aplidium nordmani* and *Botrillus leachii*. Kang *et al.*[54] isolated a unique zwitterionic benzyl hydroxyadenine, aplidiamine (4.1.6.2) from the ascidian *Aplidiopsis* sp., which was collected in Western Australia. Its structure was determined by spectral analysis and diazomethane methylated derivatives. The methyl derivatives provided additional evidence for structure of aplidiamine as the new *N*-methyl signals provided additional long range NMR heterocorrelations. Four new alkaloids, the 20-sulfated derivatives of lamellarins B (4.1.6.3), C (4.1.6.4), L (4.1.6.5) and the 8-sulfated derivatives of lamellarin G (4.1.6.6) were isolated from the ascidian *Didemnum chartaceum*, collected near Friget Cay in the Swains Reef group, Great Barrier Reef.[27]

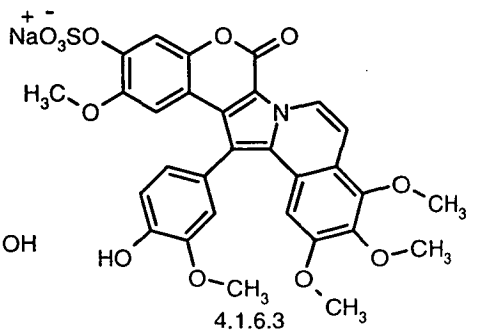
In 1999 Reddy *et al.*[55] described a new alkaloid, lamellarin α 20-sulfate (4.1.6.7) from an unidentified ascidian, collected from the Arabian Sea near Trivandrum, India. It

showed inhibitory activity against integrase, preintegration complexes (PICs) and HIV virus in cell culture.

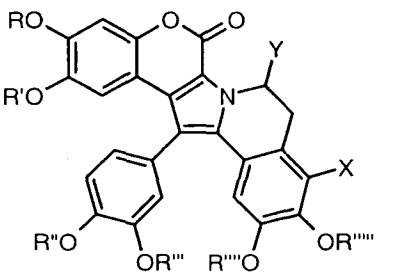


4.1.6.1

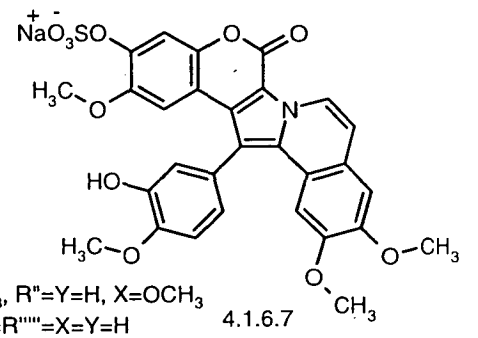
4.1.6.2



4.1.6.3

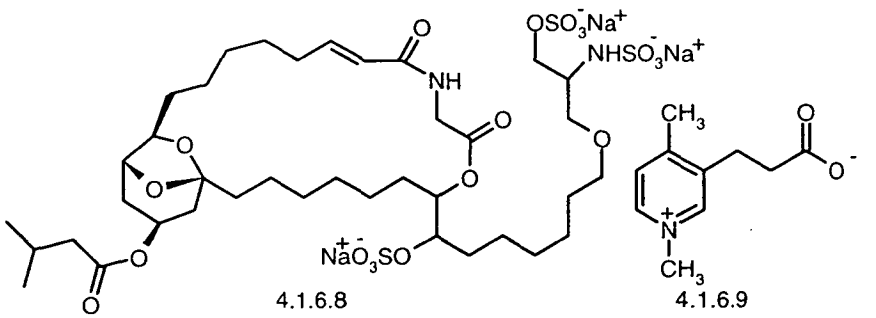


4.1.6.4 R=SO_3^-, R'=R'''=R''''=R'''''=CH_3, R''=Y=H, X=OCH_3
4.1.6.5 R=SO_3^-, R'=R''=R''''=CH_3, R'''=R'''''=X=Y=H
4.1.6.6 R=R''=R''''=CH_3, R'=R'''=X=Y=H, R'''''=SO_3^-



4.1.6.7

An inhibitor of HIV-1 integrase, cyclodidemniserinol trisulfate (4.1.6.8) was isolated from the Palauan ascidian *Didemnum guttatum*, which was collected at Ngerchaol Island.[56] Aiello *et al.*[57] isolated a new *N*-methylpyridinium alkaloid, sulcatin (4.1.6.9), from the ascidian *Microcosmus vulgaris* which was collected in the Bay of Naples (Procida, Punta Pizzaco).



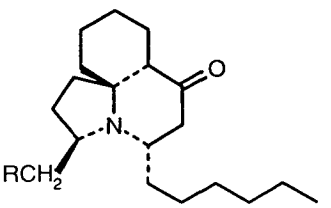
4.1.6.8

4.1.6.9

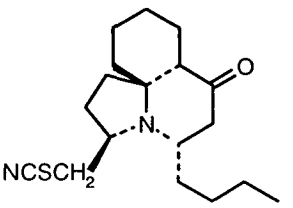
4.1.7 Miscellaneous alkaloids.

Five new perhydropyrrolo[2,1-j]quinolin-7-one alkaloids, cyclindricines C-G (4.1.7.1-4.1.7.5) were isolated from two different collections at Deep Glen Bay, East Coast,

and Bay of Islands, South Bruny Island in Tasmania of the ascidian *Clavelina cylindrica*.[58] Cyclindricines C (4.1.7.1), D (4.1.7.2) and E (4.1.7.3) were from the Deep Glen Bay collection, while cyclindricines F (4.1.7.4) and G (4.1.7.5) were from the South Bruny Island collection. This demonstrated a geographical variation in the metabolites from this species in Tasmania.

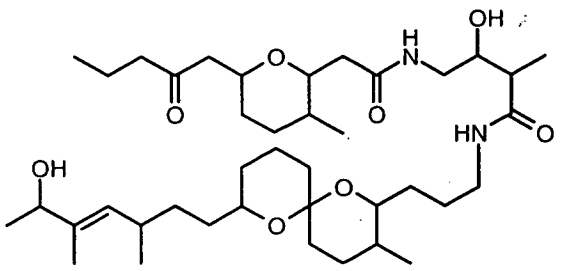


4.1.7.1 R=OH
4.1.7.2 R=OCH_3
4.1.7.3 R=$OCOCH_3$
4.1.7.4 R=SCN

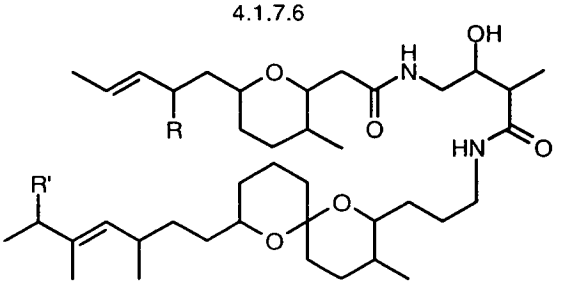


4.1.7.5

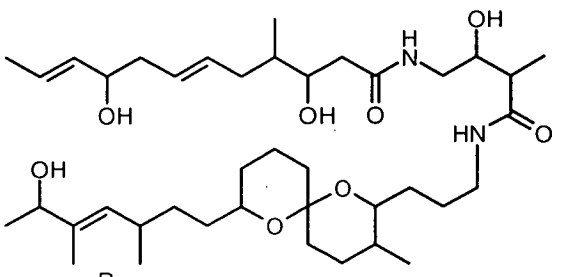
Four new cyclic polyethers, bistramides B (4.1.7.6), C (4.1.7.7), D (4.1.7.8) and K (4.1.7.9), as well as the known bistramide A (4.1.7.10) were isolated from the ascidian *Lissoclinum bistratum*, which was collected near Ua and N'Do Islands, on the south coast of New Caledonia.[59] McDonald *et al.*[60] isolated new brominated tyrosine derivatives, botryllamides A-D (4.1.7.11-4.1.7.14) from the styelid ascidian *Botryllus schlosseri*, which was collected from Little Trunk Reef, Great Barrier Reef, Australia and from a *Botryllus* sp., collected from Siquijor Island, Philippines. A glycosphingolipid, cerebroside (4.1.7.15) was isolated from the *n*-butanol extract of *Botrillus leachii* as its peracetate (4.1.7.16).[49] The ascidian was collected in the Lagoon of Venice, Italy.



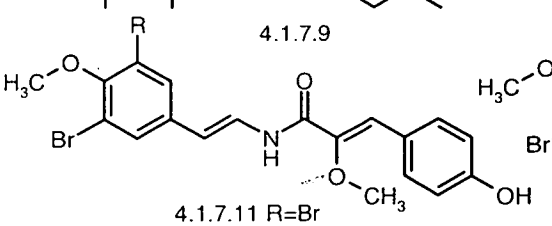
4.1.7.6



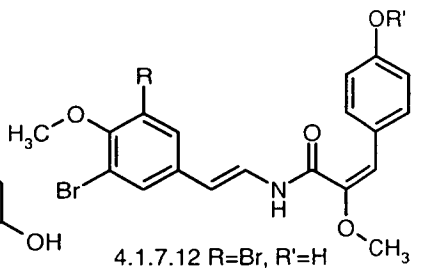
4.1.7.7 R=R'=O
4.1.7.8 R=R'=OH
4.1.7.10 R=O, R'=OH



4.1.7.9

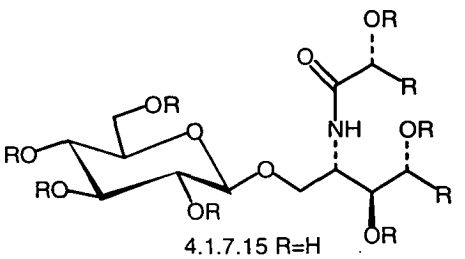


4.1.7.11 R=Br
4.1.7.13 R=H

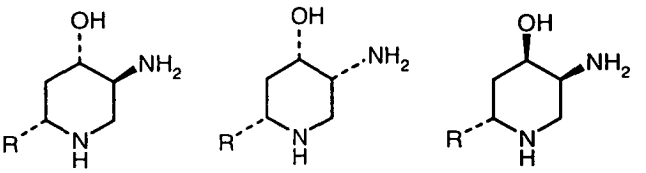


4.1.7.12 R=Br, R'=H
4.1.7.14 R=R'=H

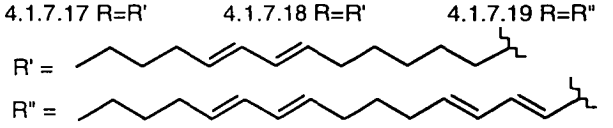
Freyer *et al.*[61] reported three new piperidine alkaloids, pseudodistomins D-F (4.1.7.17-4.1.7.19) from a bioassay-guided fractionation of the methanol-dichloromethane extract of the ascidian *Pseudodistoma megalarva*, which was collected in the Rock Islands, Palau. A high throughput screen to evaluate natural products in a yeast-based assay for DNA damaging activity was initiated because effective antitumour agents can act through many mechanisms which result in DNA damage. The ascidian extract showed differential activity in a DNA repair-deficient yeast mutant and was thus selected for fractionation.



4.1.7.15 R=H
4.1.7.16 R=Ac

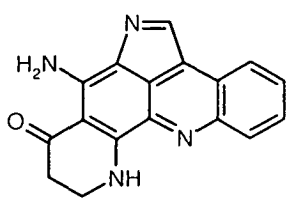


4.1.7.17 R=R' 4.1.7.18 R=R' 4.1.7.19 R=R"

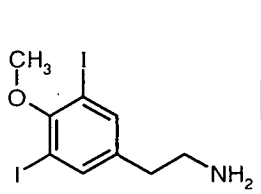


A new pyrroloacridine alkaloid, plakindine D (4.1.7.20) and a known metabolite, 3,5-diiodo-4-methoxyphenethylamine (4.1.7.21) were isolated from the ascidian *Didemnum rubeum*.[62] The specimen was collected near the island of Rota, Northern Mariana Islands.

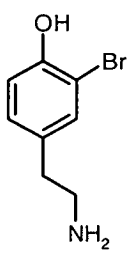
A new metabolite, 2-(3'-bromo-4'-hydroxyphenyl)ethanamine (4.1.7.22) was isolated from the New Zealand ascidian *Cnemidocarpa bicornuta*.[63]



4.1.7.20

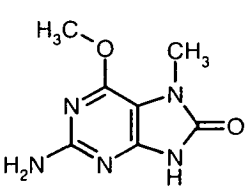


4.1.7.21

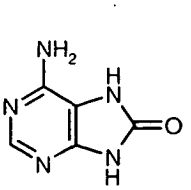


4.1.7.22

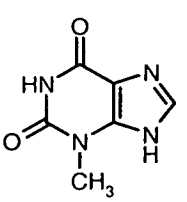
In 1999 Lindsay *et al.*[64] isolated an unusual purine, 6-methoxy-7-methyl-8-oxoguanine (4.1.7.23) and two known compounds, 8-oxoadenine (4.1.7.24) and 3-methylxanthine (4.1.7.25) from the ascidian *Symplegma rubra* collected on the southeastern coastline of Brazil.



4.1.7.23



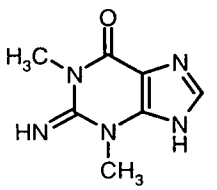
4.1.7.24

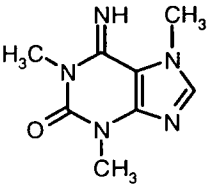


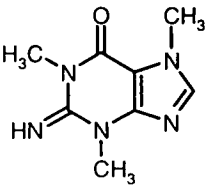
4.1.7.25

A new purine, (1,3-dimethylguanine (4.1.7.26) was isolated from the New Zealand ascidian *Botrylloides leachi*, collected in Momorangi Bay, Marlborough Sounds.[65]

Copp *et al.*[66] isolated a new purine, 1,3,7-trimethylisoguanine (4.1.7.27) from the ascidian *Pseudodistoma cereum*, which was collected from Irishman's Garden in the Three Island Group, New Zealand. Its structure was based on NMR spectroscopic and mass spectrometric data and by comparison with the known purine (4.1.7.28).

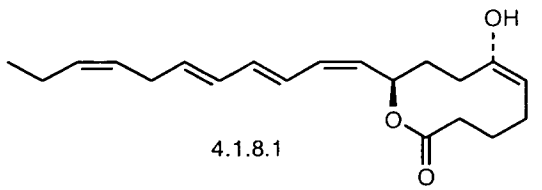

4.1.7.26

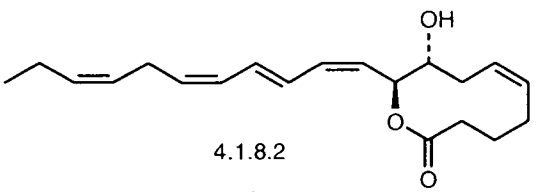

4.1.7.27

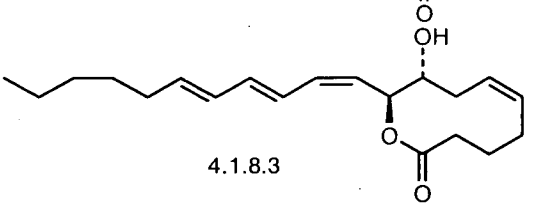

4.1.7.28

4.1.8 Miscellaneous non-nitrogenous compounds.

Three new fatty acid metabolites, didemnilactones A (4.1.8.1), B (4.1.8.2) and neodidemnilactone (4.1.8.3) were isolated from the ascidian *Didemnum moseleyi* (Herdman) by Niwa *et al.*[67] The specimen was collected in Ago Bay, Mie Prefecture, Japan.

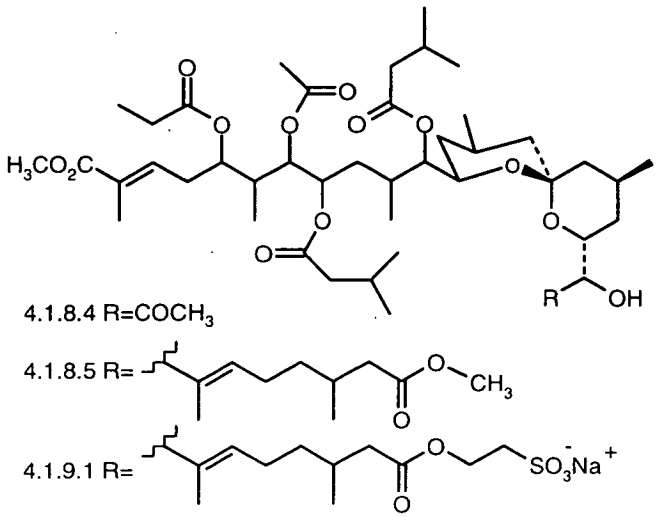

4.1.8.1


4.1.8.2


4.1.8.3

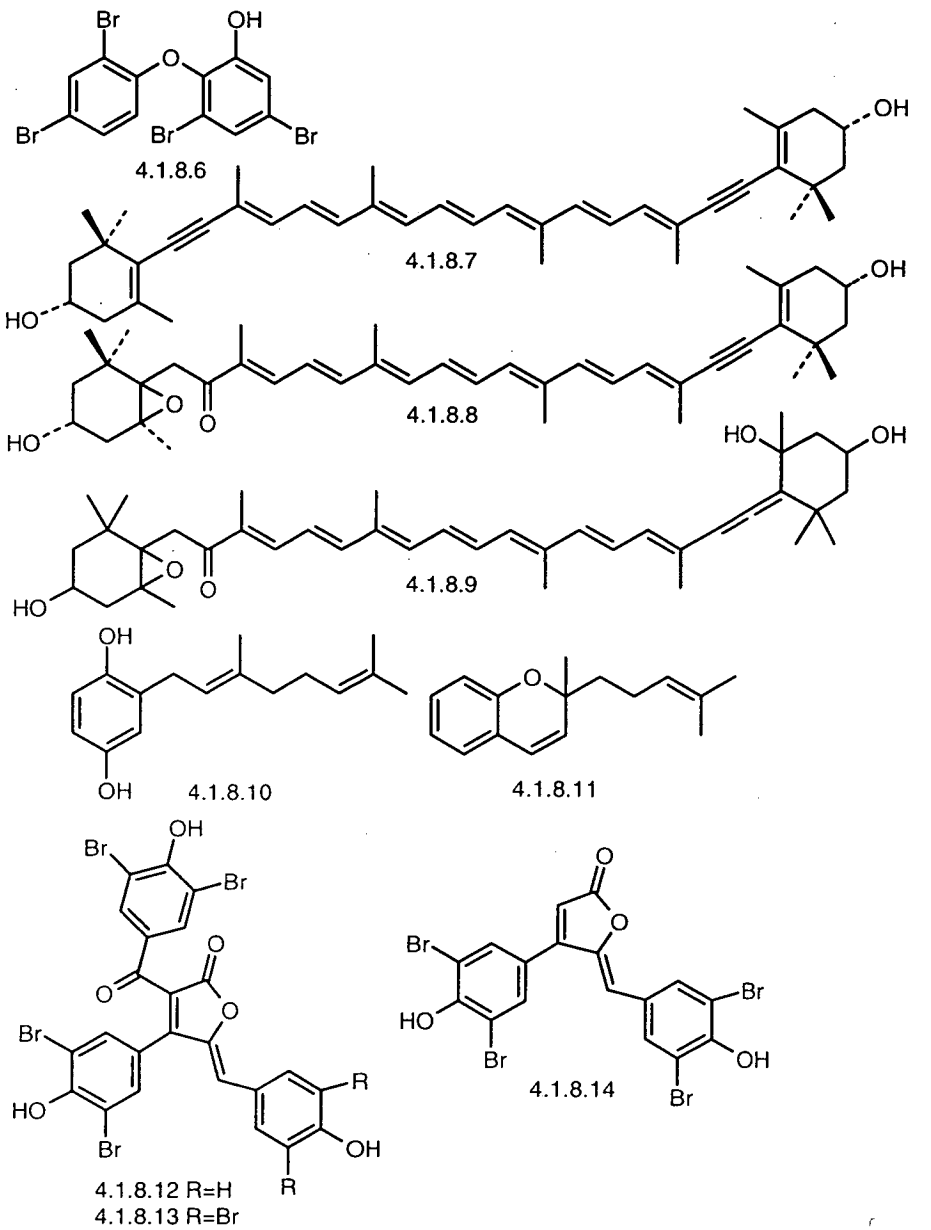
Pika *et al.*[68] reinvestigated the ascidian *Didemnum* sp. from Palau and revealed that the previously reported metabolites, didemnaketals A (4.1.8.4) and B (4.1.8.5) were artifacts from a prolonged storage of the ascidian in methanol. Since neither didemnaketals A and B were present in new species of the *Didemnum* sp. that were collected in exactly the same location as before. Fresh extract gave a terpenoid, didemnaketal C (4.1.9.1) whose

methanolysis afforded didemnaketal B. While didemnaketal A was presumably an autoxidation product.



A known compound, 2-(2',4'-dibromophenoxy)-3,5-dibromophenol (4.1.8.6) was isolated from the ascidian *Didemnum* sp. by Schumacher *et al.*[36]

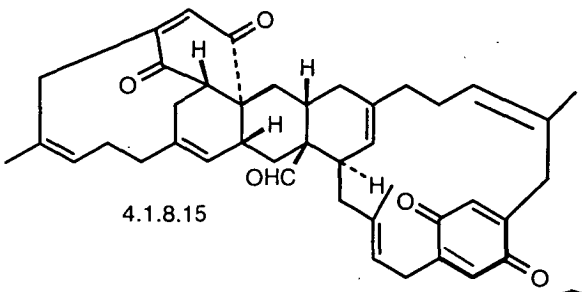
Aiello *et al.*[57] reported two new carotenoid pigments (4.1.8.7, 4.1.8.8) and a known fucoxanthinol (4.1.8.9), together with two known terpene hydroquinones, geranylhydroquinone (4.1.8.10) and 2-methyl-2-(4-methylpent-3-enyl)-2*H*-chromen-6-ol (4.1.8.11) from the ascidians *Aplidium nordmani* and *Styela partita*, collected in the Lagoon of Venice, Italy.



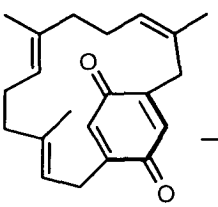
In 1998 two new non-nitrogenous metabolites, cadiolides A (4.1.8.12), B (4.1.8.13) and known rubrolide A (4.1.8.14) were isolated as amorphous solids from an ascidian *Botryllus* sp. after purification through Sephadex LH-20 and gradient silica flash chromatography. The specimen was collected at Barrang Caddi, Indonesia. The structures were determined using ES-MS, MALDI-MS and 2D NMR spectroscopy.[69]

Eleven new cyclofarnesylated quinone derived metabolites, longithorones A-I (4.1.8.15-4.1.8.23)[70, 71] and J-K (4.1.8.25-4.1.8.26)[72] were isolated from the ascidian *Aplidium longithorax*, collected in Palau and Gannet Cay, at the Swains Reefs,

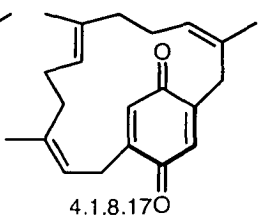
respectively. Two known bromodiphenyl ethers (4.1.8.6, 4.1.8.24)[58] were also isolated from the Palau ascidian.



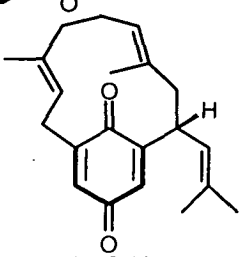
4.1.8.15



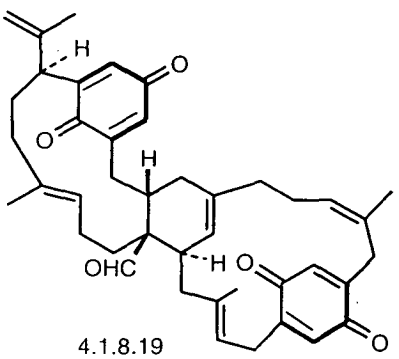
4.1.8.16



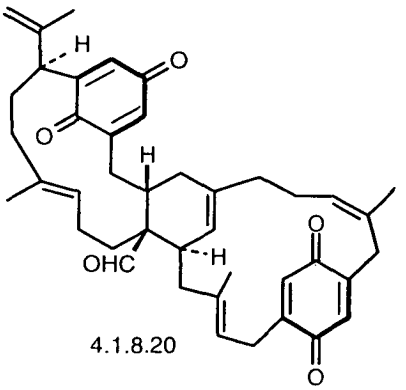
4.1.8.17



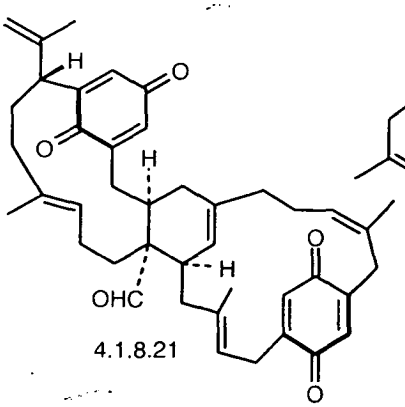
4.1.8.18



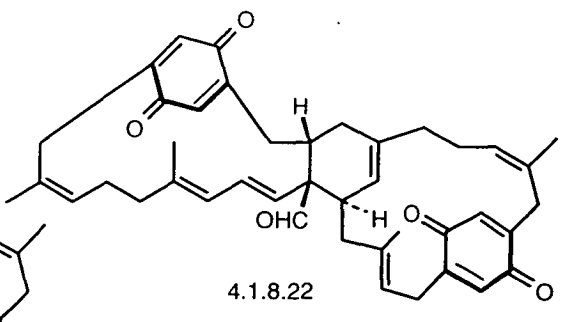
4.1.8.19



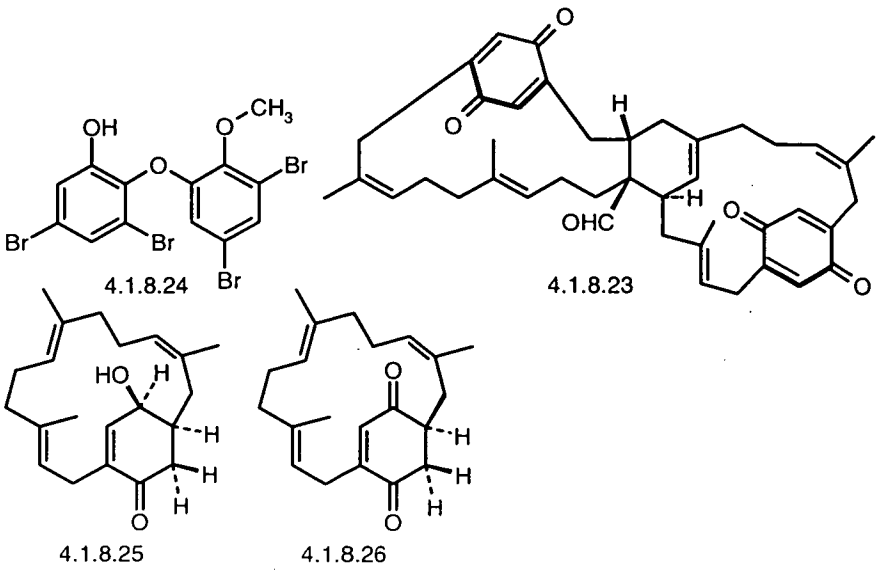
4.1.8.20



4.1.8.21

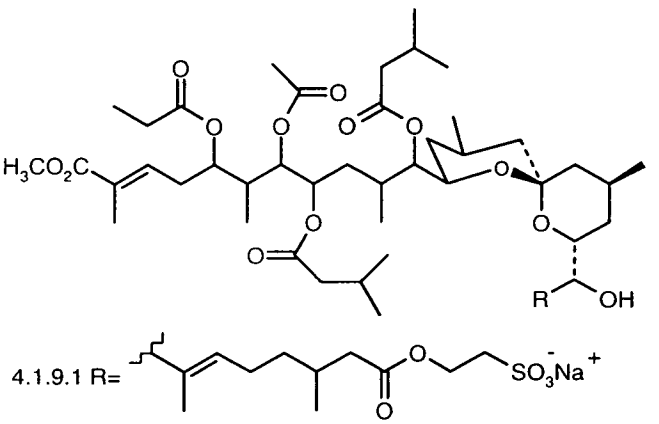


4.1.8.22

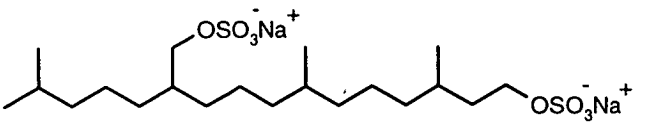


4.1.9 Non-nitrogenous salt metabolites.

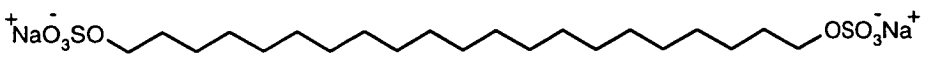
The terpenoid didemnaketal C (4.1.9.1), which was isolated as a sodium sulfate salt from a reinvestigation of the ascidian *Didemnum* sp. from Palau, did not inhibit HIV-1 protease in a peptidolysis assay.



Two sodium sulfate salts, 3,7,11,15-tetramethyl-hexadecan-1,19-sodium disulfate (4.1.9.2) and heneicosane-1,21-sodium disulfate (4.1.9.3) were isolated from the ascidian *Ascidia mentula*, which was collected in Corigliano Gulf, Ionian Sea, Southern Italy.[73] Both metabolites were tested for an antiproliferative activity on IGR1 human melanoma, J774 murine monocyte/macrophage, WEHI164 murine fibrosarcoma and P388 murine leukemia cell line *in vitro*. Both compounds inhibited the growth of all cell lines evaluated at 96 hours.

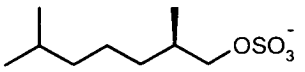


4.1.9.2

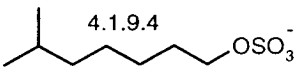


4.1.9.3

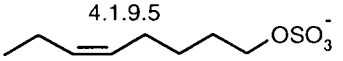
One known alkane, (*R*)-2,6-dimethylheptyl sulfate (4.1.9.4), and two new metabolites, 6-methylheptyl sulfate (4.1.9.5) and (*E*)-5-octenyl sulfate (4.1.9.6), were isolated from the Mediterranean ascidian *Halocynthia papillosa*. The specimen was collected in the Corigliano Gulf (Ionian Sea, Southern Italy).



4.1.9.4



4.1.9.5



4.1.9.6

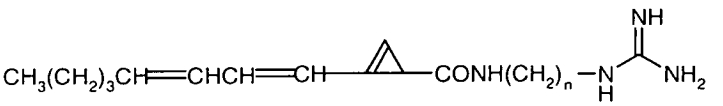
From 1994 to 2000, marine ascidians afforded a variety of secondary metabolites. Cyclic peptides and macrocyclic alkaloids were the most frequently reported compound classes, representing about 25% of all ascidian secondary metabolites. The rest were the other amino acid derived compounds, steroidal alkaloids, non-nitrogenous compounds and the zwitterionic compounds. The zwitterionic and the salt metabolites were the least percentage recorded, around 3-4% of all metabolites from ascidians.

4.2 General introduction and secondary metabolites from ascidians *Polyandrocarpa* sp.

The ascidian genus *Polyandrocarpa* is found worldwide and occurs in both temperate and tropical waters. *Polyandrocarpa lapidosa* (Herdman) in this study belongs to Phylum Chordata, Sub-Phylum Tunicata or Urochordata, class Ascidiacea, order Pleurogona, suborder Stolidobranchia, family Styelidae, and subfamily Polyzoinae. *Polyandrocarpa lapidosa* occurs as large colonial encrustations, bright reddish-brown to orange in colour.[1]

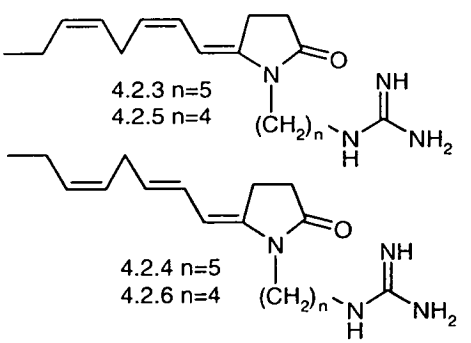
The first report of antimicrobial activity from a tunicate *Polyandrocarpa* sp. was published in 1972. The activity was against *Staphylococcus aureus*. The specimens were collected from the Gulf of California.[74]

In 1978, a *Polyandrocarpa* sp., which was collected at Bahia de Los Angeles in Baja California, was investigated. The authors isolated polyandrocarpidine I (4.2.1) and polyandrocarpidine II (4.2.2) from this ascidian. A chloroform soluble fraction of an ethanol extract was chromatographed on a Si gel column. A mixture of polyandrocarpidine I (90%) and polyandrocarpidine II (10%) was eluted from chloroform-methanol (3:2). The mixture inhibited *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Mycobacterium avium*. In addition, it was cytotoxic toward monkey kidney tissue culture cells and showed slight antiviral activity against *Herpes* virus.[75]



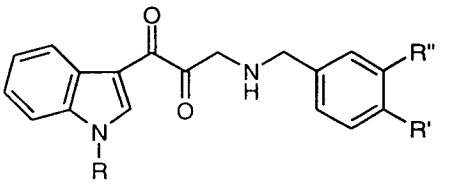
4.2.1 n=5, 4.2.2 n=4

Four years later, several specimens of *Polyandrocarpa* sp. were examined. An ethyl acetate soluble material from a methanolic extract was chromatographed on Si gel and Sephadex LH-20 using methanol-dichloromethane (1:1) as eluent. The antimicrobial fractions were analyzed by HPLC on C-18 porasil using 25% acetonitrile and 75% 0.05 M aqueous phosphate buffer (pH 5) containing 0.005 M tetrabutylammonium bisulfate. Polyandrocarpidines A (4.2.3), B (4.2.4), C (4.2.5) and D (4.2.6) were isolated. The authors claimed that polyandrocarpidine B (4.2.4) was a revised structure of polyandrocarpidine I (4.2.1) and polyandrocarpidine D (4.2.6) was a revised structure of polyandrocarpidine II (4.2.2).[76]



In 1983 the structures of polyandrocarpidines I (4.2.1) and II (4.2.2) were revised to structures (4.2.4) and (4.2.6), respectively and the compound (4.2.4) was synthesised by Rinehart *et al.*[77]

In 1990 four new indole-derived metabolites, polyandrocarpamides A (4.2.7), B (4.2.8), C (4.2.9), and D (4.2.10) were isolated from the ascidian *Polyandrocarpa* sp., which was collected at Siquijor Island, the Philippines. The acetone extract was fractionated by vacuum flash chromatography on Si gel and purified by reversed phase HPLC (ODS silica) using methanol-water (7:3) as eluent.[78]



4.2.7 R=H, R'=OH, R"=Br
4.2.8 R=H, R'=OH, R"=I
4.2.9 R=H, R'=OH, R"=H
4.2.10 R=R'=R"=H

4.3 Results and discussion.

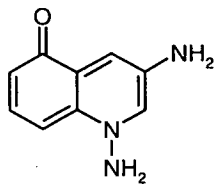
Polyandrocarpa lapidosa was collected at Spikey Bridge, East Coast of Tasmania by scuba diving. Freeze-dried *Polyandrocarpa lapidosa* was extracted with methanol until showing negative to Meyer's reagent. The concentrated methanol extract and the solid residue were each separately partitioned between 2-butanol and water. The 2-butanol soluble material was positive to Meyer's test, as opposed to the aqueous layer which was negative and discarded. A preliminary investigation was performed in order to know whether or not there were some alkaloid metabolites in ethyl acetate, dichloromethane, and petroleum soluble fractions. Most of them were fatty acids, and derivatives of sterols. Consequently the combined 2-butanol soluble material was subjected twice to a C18 column chromatography with increasing percentage of methanol in water until 100% MeOH. Then the fractions which showed a positive test to Meyer's reagent were purified further several times on a Sephadex LH-20 column with MeOH as eluent.

The first fraction was eluted from both the C18 column with 10-20% MeOH-water and a Sephadex LH-20 column with methanol as a white and hydroscopic solid. These properties implied a polar material. The dry solid was water-soluble giving a pH about neutral or very weak acid (pH~6-7). After heating the dry solid (weight of 0.0025 g starting

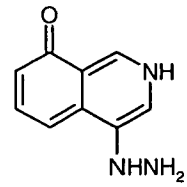
material) to high temperature a white residue was left that formed a very strongly basic aqueous solution (pH~14). This indicated a possible ionic compound. However, the weight of the dry residue (four repetitions) after heating was observed as 0.0002, 0.003, 0.0005 and 0.0005 g. Elemental analysis result was not promising (found C 1.21, H 5.81 and N 0.14). Both heating weight loss and elemental analysis results were not consistent. Could it be possible that the solid could be a mixture of salts? It could not be sodium chloride salt.

The second fraction eluting from the Sephadex LH-20 column contained an unidentified alkaloid whose molecular formula possibly was $C_{17}H_{24}N_2O_6$ (equivalent to its molecular weight of 352). However, a small peak of 541 was also shown in both LSIMS (Figure 4.3.1) and ESIMS. Therefore, either 352 or 541 or higher number was its molecular weight. The ^{13}C NMR spectrum (Figure 4.3.2) and the ^{1}H NMR spectrum (Figure 4.3.3) did not allow a structure to be proposed. This compound was isolated as a pale yellow oil. Unfortunately, time was limited to purify and complete the identification of the structure.

The third fraction yielded an unidentified alkaloid whose molecular weight of 175 analyzed from both LSIMS (Figure 4.3.4) and EIMS (Figure 4.3.5). It was purified by Sephadex LH-20 and isolated as yellow oil. The molecular formula of the alkaloid was $C_9H_9N_3O$, corresponded to seven degrees of unsaturation. The ^{13}C NMR spectrum (Figure 4.3.6) and DEPT experiment (Figure 4.3.7) showed one carbonyl carbon at 158.0 ppm, the other three aromatic quaternary carbons at 147.6, 128.0, and 119.0 ppm, together with five aromatic methine carbons at 130.7, 128.0, 126.1, 115.9 and 106.7 ppm. The ^{1}H NMR spectrum (Figure 4.3.8) showed chemical shifts at 6.73 (s), 6.83 (d, *J* 8.8 Hz), 7.08 (dd, *J* 8.8, 14.8 Hz), 7.31 (d, *J* 8.8 Hz) and 7.52 (s) ppm. Although indoles have been previously found in *Polyandrocarpa* sp. this spectral information is not consistent with that compound type but rather suggests another *N*-heteroaromatic fused bicyclic compound. Either a quinolinone or isoquinolinone derivative is more likely, for example, compound (4.3.1) or an isomer of it, otherwise compound (4.3.2) or an isomer of it. However, it could have two or three nitrogens incorporated in (6, 6) aromatic rings; it could even be (6, 5) *N*-heteroaromatic fused bicyclic compound. Unfortunately, time was limited to complete the identification of the structure. However, this is an interesting compound worthy of further study.



4.3.1

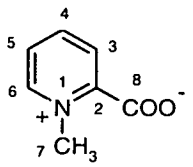


4.3.2

The fourth fraction yielded homarine (4.1.6.1), which was isolated as very fine transparent needles. Synthetic homarine was made and shown to be identical to the natural product by ^{1}H NMR spectroscopy.

4.3.1 The structure of homarine (4.1.6.1).

The LSIMS and ESI LCMS both gave a pseudo-molecular ion, $[M+H]^{+}$ at m/z 138 corresponding to a molecular formula of $C_7H_7NO_2$, indicating five degrees of unsaturation. The ^{13}C NMR spectrum (Figure 4.3.1.1, CD_3OD as solvent, Table 4.3.1.1) showed seven carbons consistent with the formula. The ^{1}H NMR spectrum (Figure 4.3.1.2, CD_3OD as solvent, Table 4.3.1.1) showed four aromatic protons and a methyl group attached to nitrogen. Synthesis of homarine from a reaction of picolinic acid and methyl iodide[79] was performed in this study to confirm the identification. Homarine had been previously found from various marine sources, such as, the hydroids *Tubularia larynx*,[80] and *Hydractinia echinata*,[81] the ascidian *Halocynthia roretzi*,[82, 83] and the sponges *Spongosorites* sp.[84] and *Cymbastela cantharella*.[85] It is the first time that homarine has been isolated from the ascidian *Polyandrocarpa lapidosa* from this study. Urban *et al.*[84] reported that a crude ethanol extract of the *Spongosorites* sp. displayed significant antibiotic activity against both Gram-positive and Gram-negative bacteria. The known marine metabolite homarine was detected in the crude ethanol extract.[84]



4.1.6.1

Table 4.3.1.1 ^{1}H and ^{13}C NMR data of homarine (4.1.6.1), CD_3OD as solvent, and the synthetic homarine, CD_3OD-$CDCl_3$ as solvent.

No.	Homarine		Synthetic homarine
	^{1}H, *J* (Hz)	^{13}C	^{1}H, *J* (Hz)
1	-	-	-
2	-	126.39	-
3	8.79 d, 6.0	126.41	9.05 d, 5.2
4	7.94 t, 6.4	145.9	8.31 t, 6.4
5	8.52 t, 6.7	145.4	8.77 dt, 1.6, 8.0
6	8.08 d, 8.0	154.2	8.57 d, 8.0
7	4.42 s	47.4	5.24 s
8	-	164.0	-

aei0027 Scan 1 (Av 3-23 Acq) 100%=114239 mv 14 Nov 100 14:39
LRP +LSIMS Aei 16D129A1 LSIMS in mnba

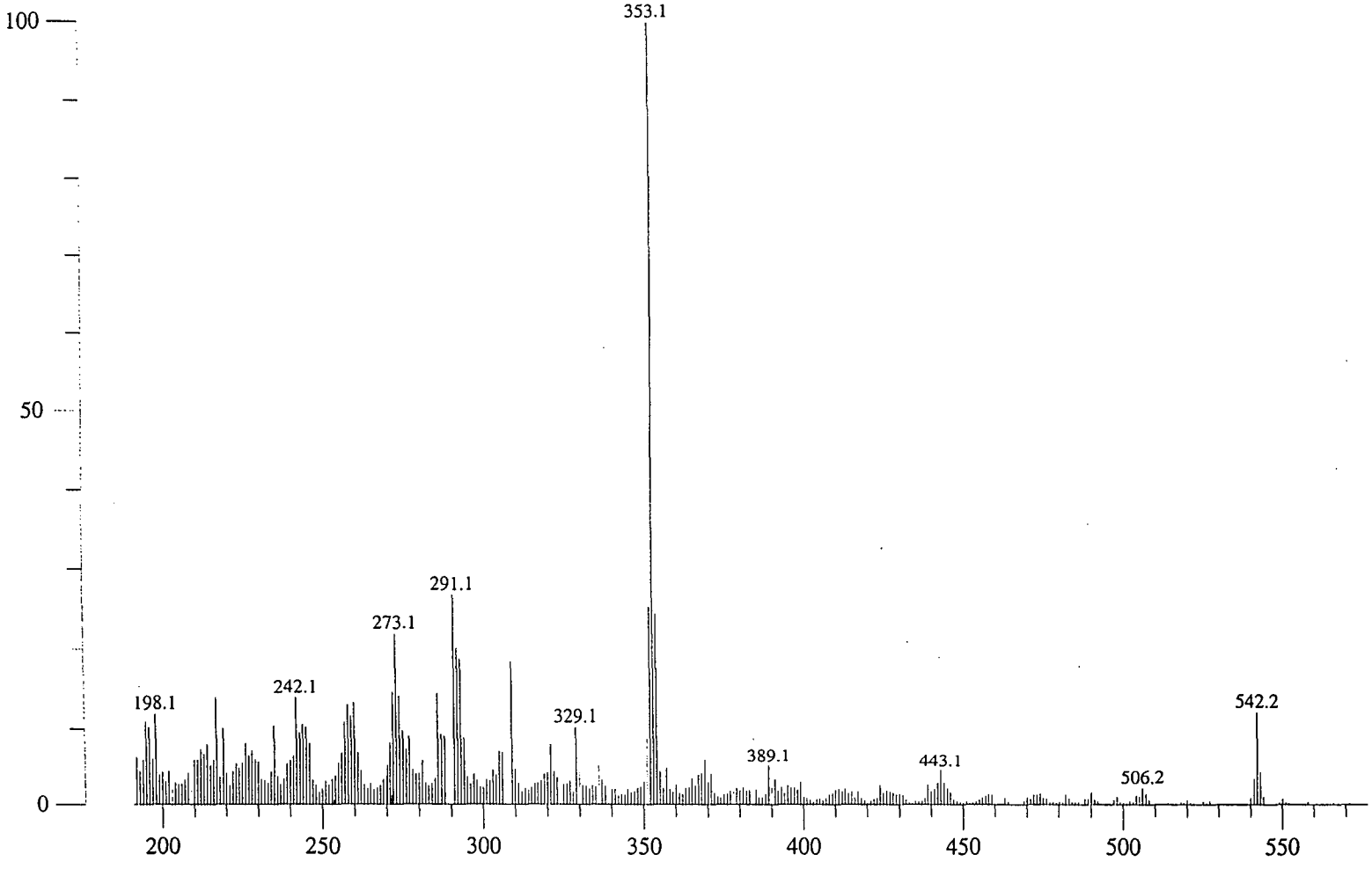


Figure 4.3.1 LSIMS data of the alkaloid MW 352 or 541.

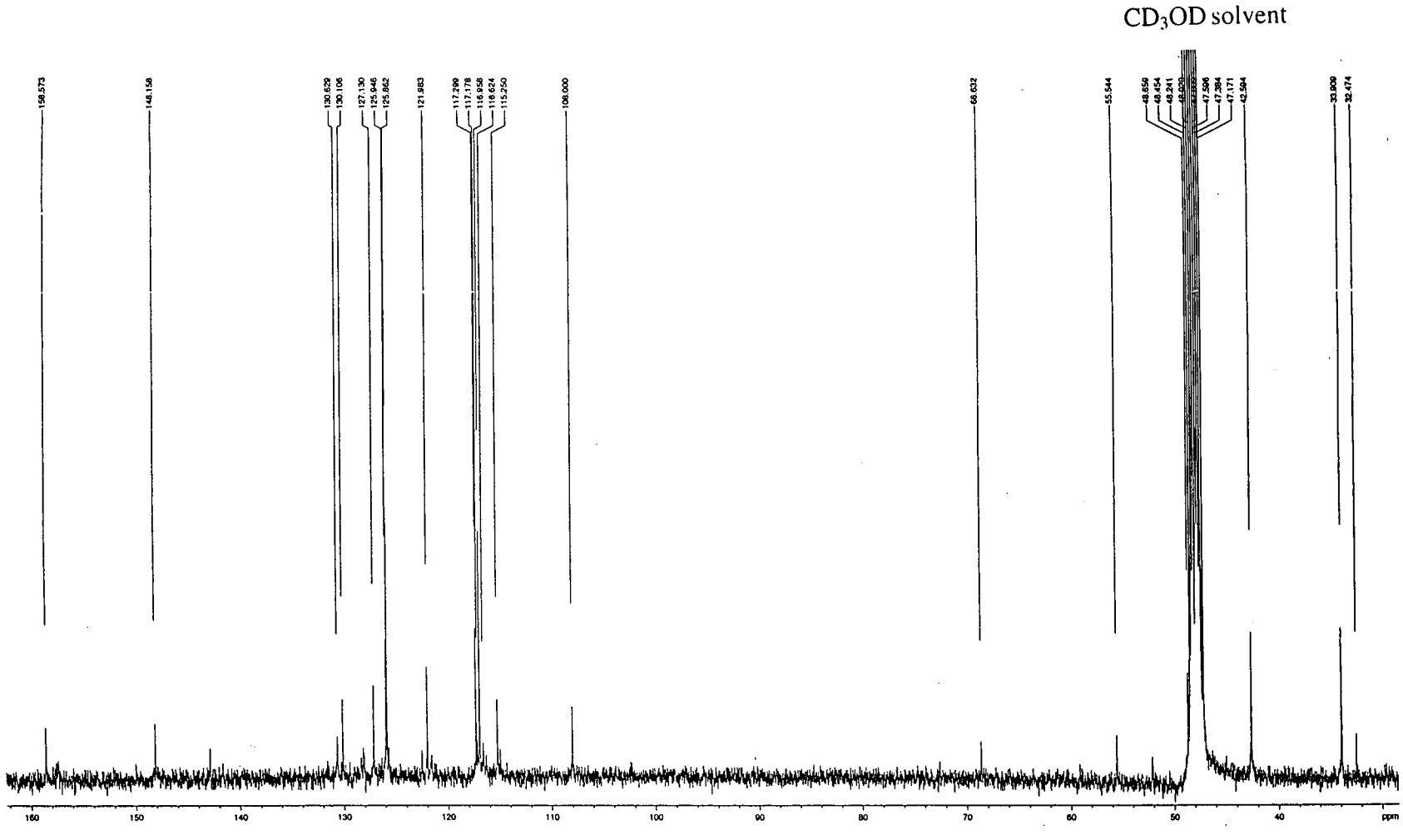


Figure 4.3.2 ^{13}C NMR spectrum of the alkaloid MW 352 or 541.

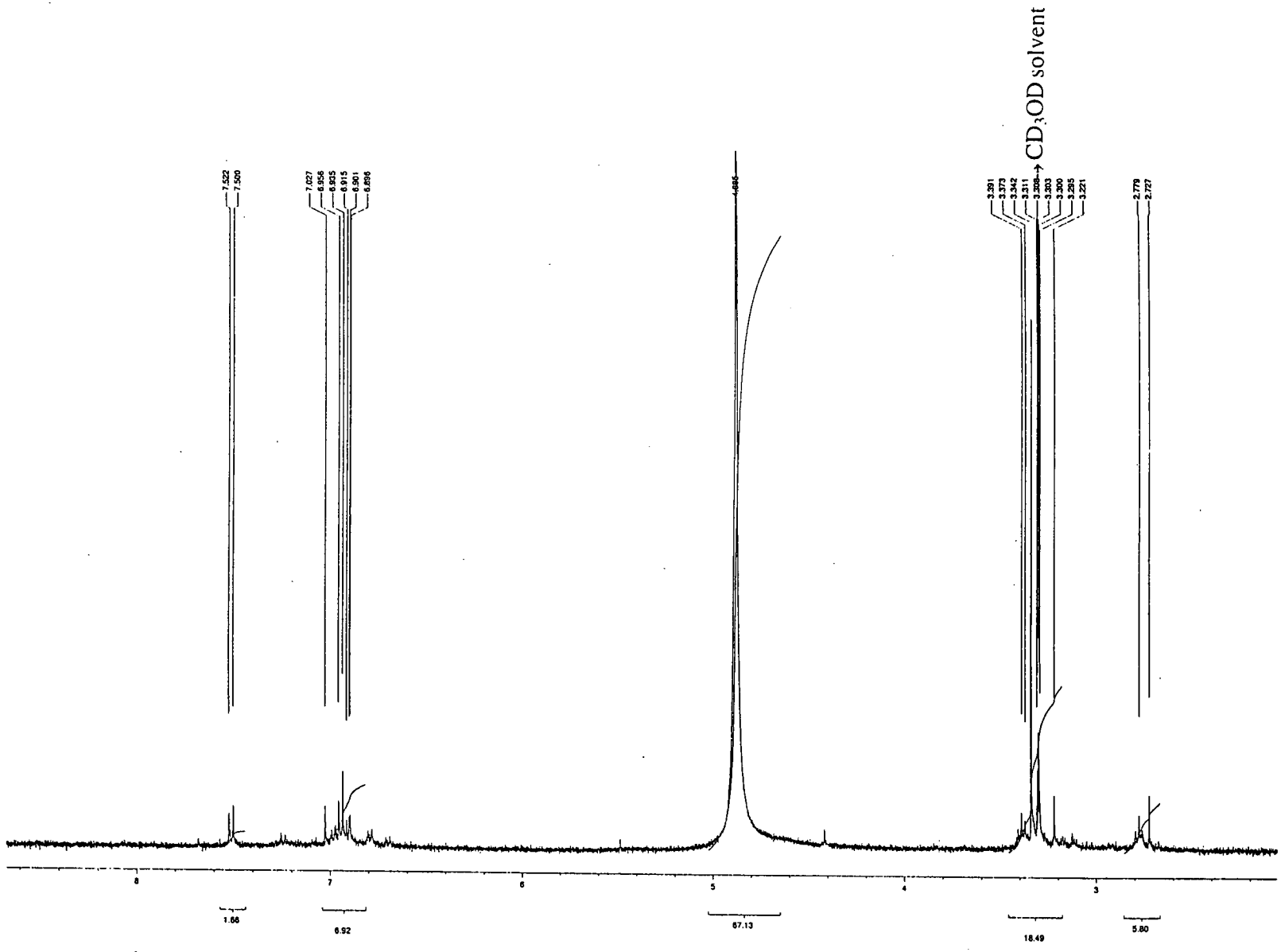


Figure 4.3.3 ^{1}H NMR spectrum of the alkaloid MW 352 or 541.

adel0005 Scan 1 (Av 2-23 Acq) 100%=1119152 mv 22 Aug 100 14:27
LRP +LSIMS Aei 16D99C LSIMS in mnba

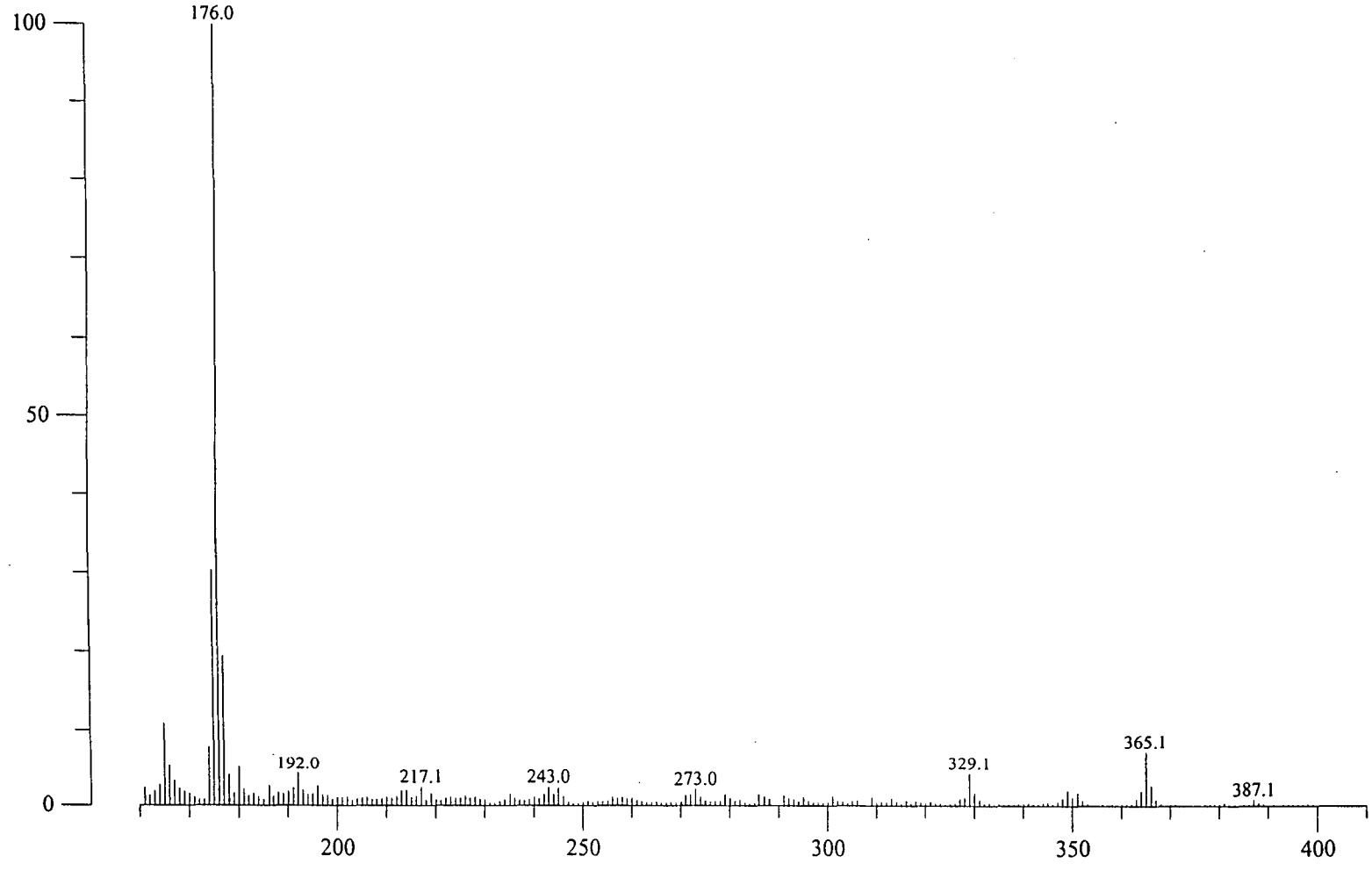


Figure 4.3.4 LSIMS data of the alkaloid MW 175.

aei0011 Scan 1 (Av 38-53 Acq) 100%=108479 mv 16 Aug 100 15:51
LRP +EI Aei 16D99C EI

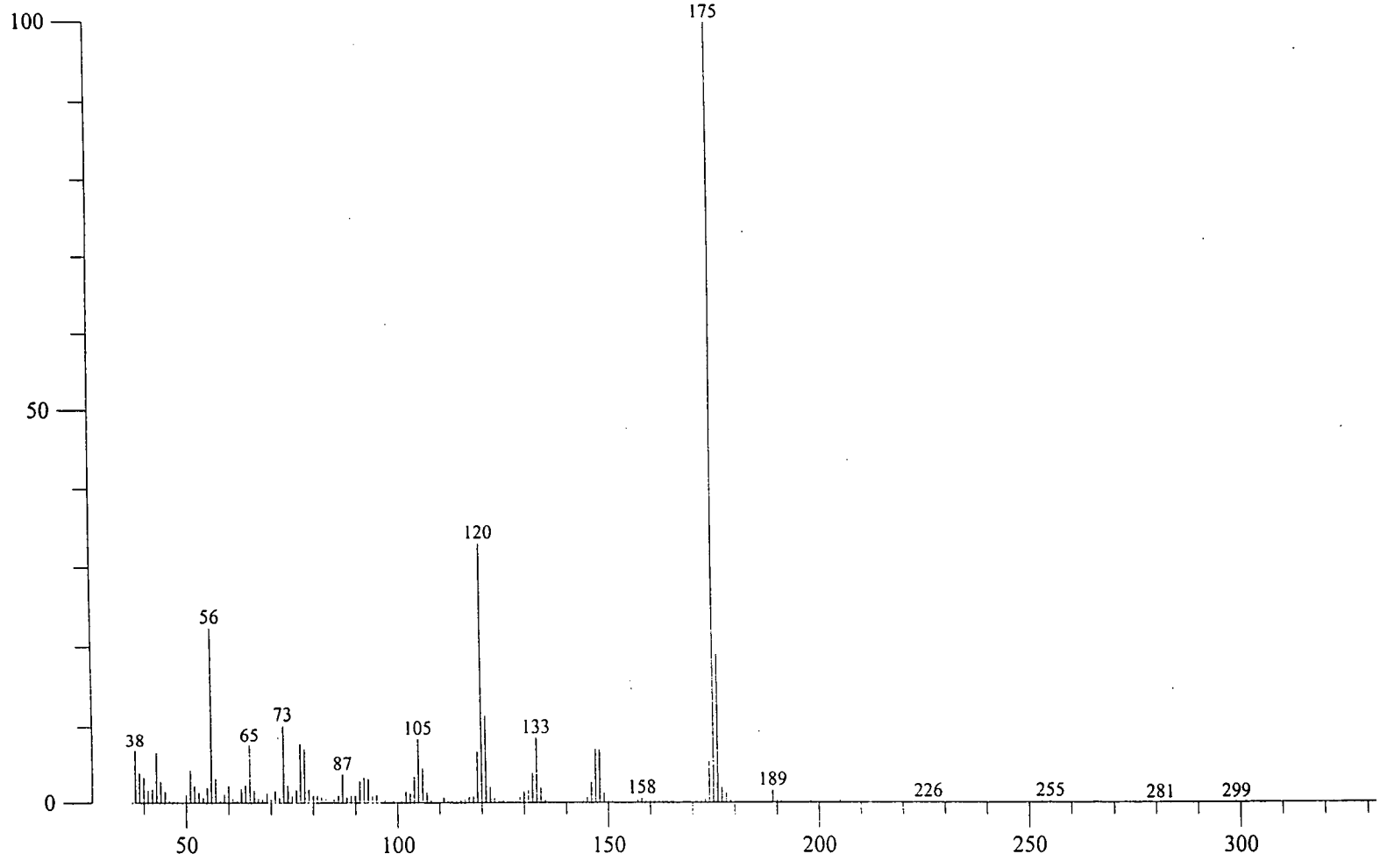


Figure 4.3.5 EIMS data of the alkaloid MW 175.

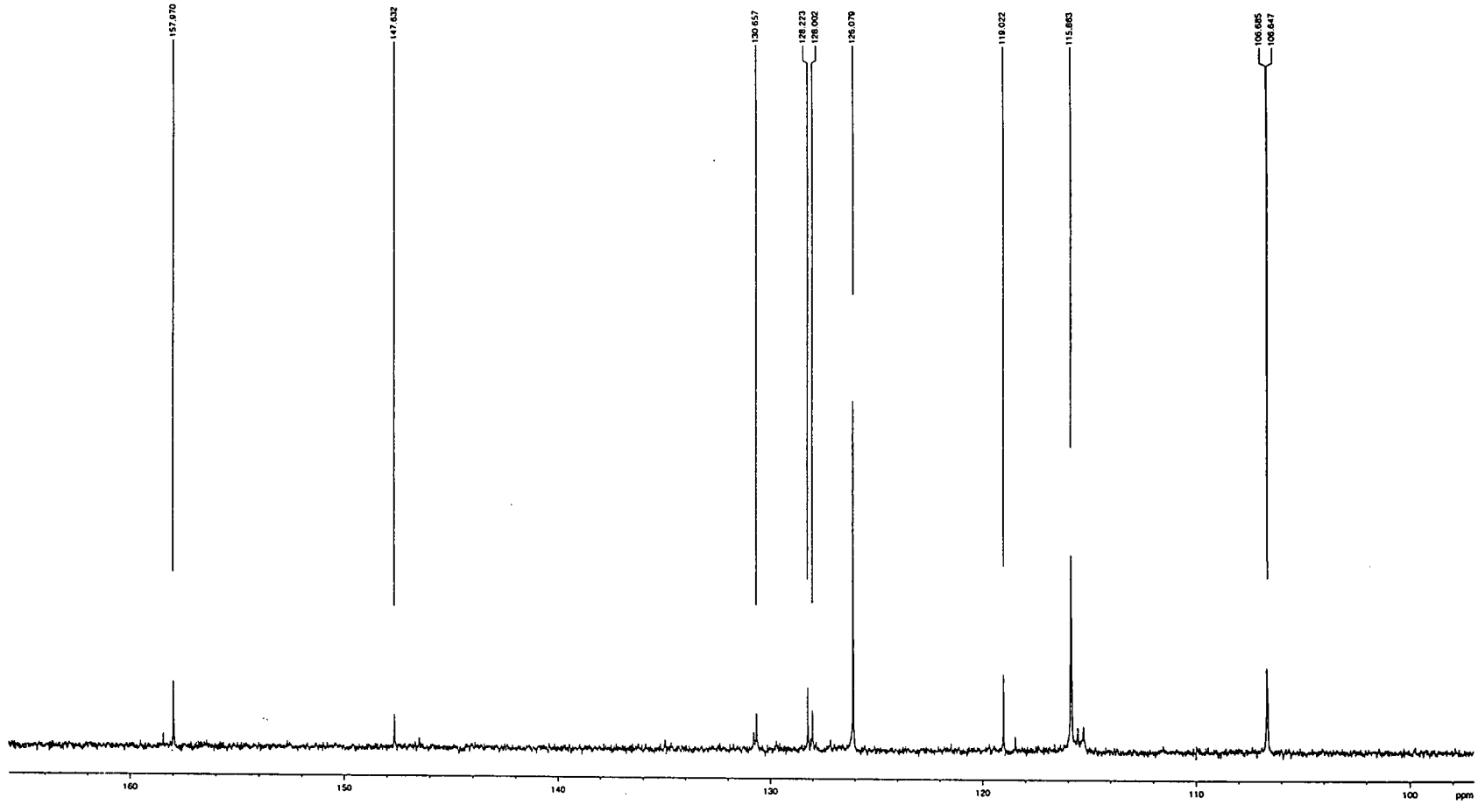


Figure 4.3.6 ^{13}C NMR spectrum of the alkaloid MW 175.

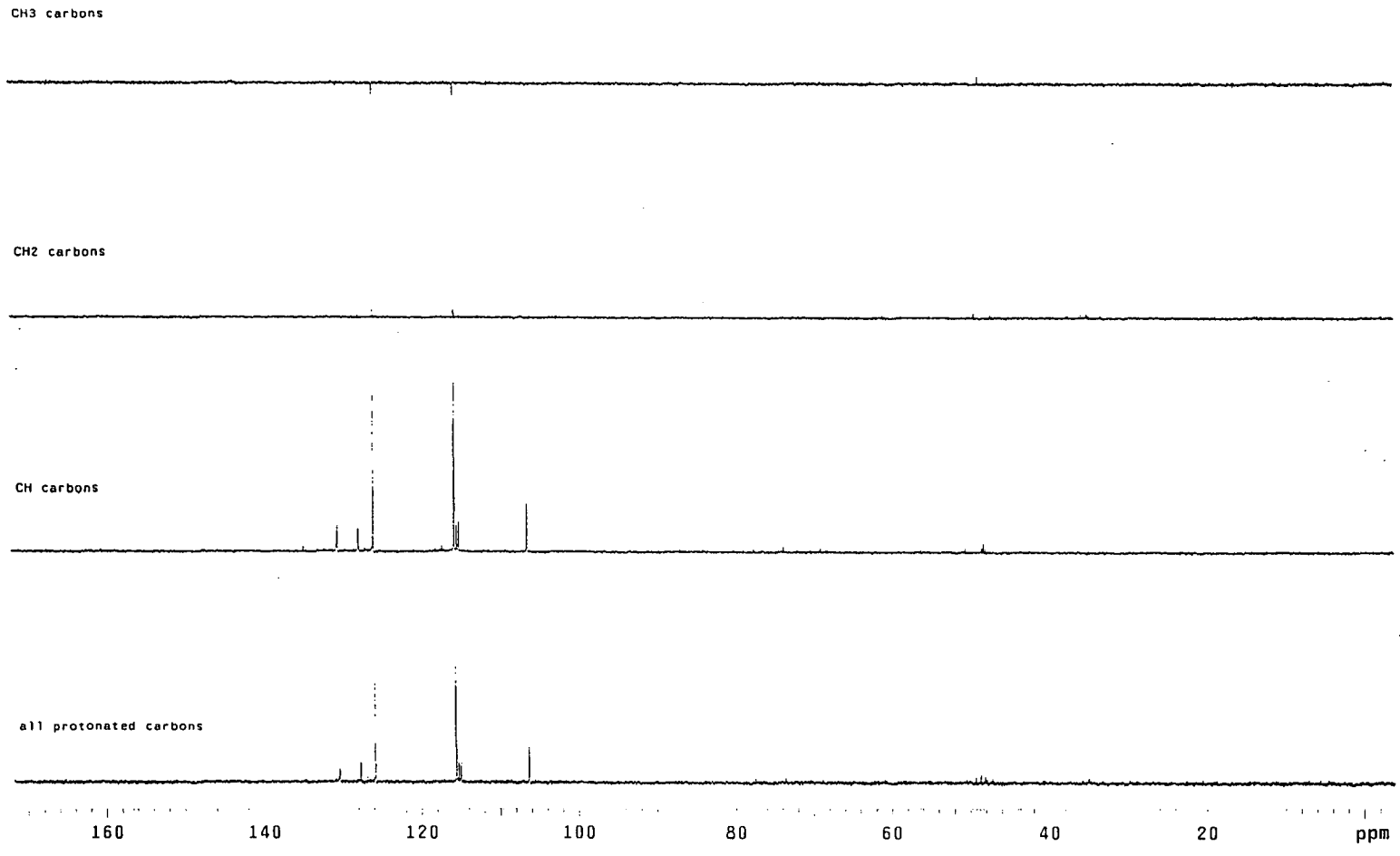


Figure 4.3.7 DEPT experiment of the alkaloid MW 175.

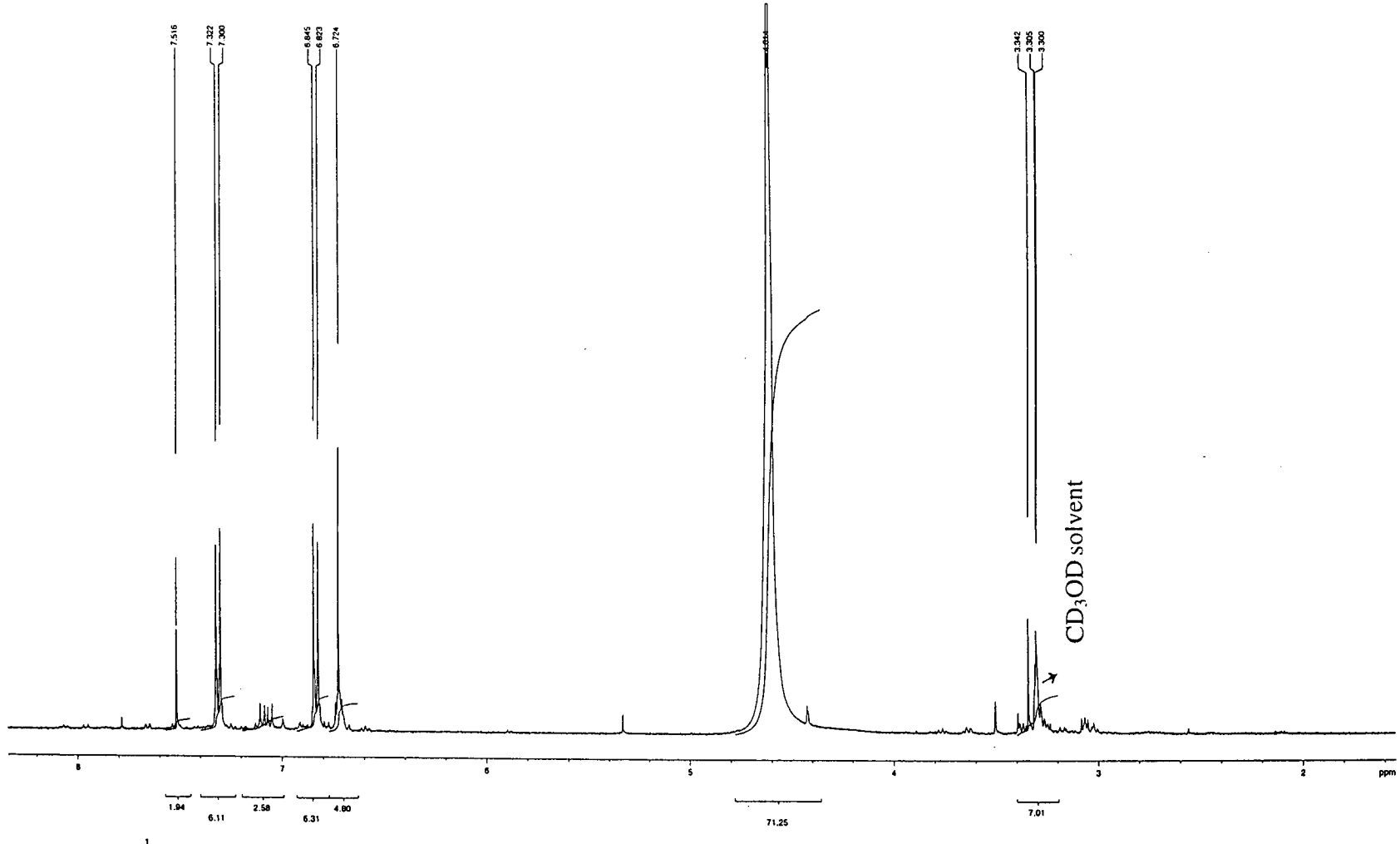


Figure 4.3.8 ^{1}H NMR spectrum of the alkaloid MW 175.

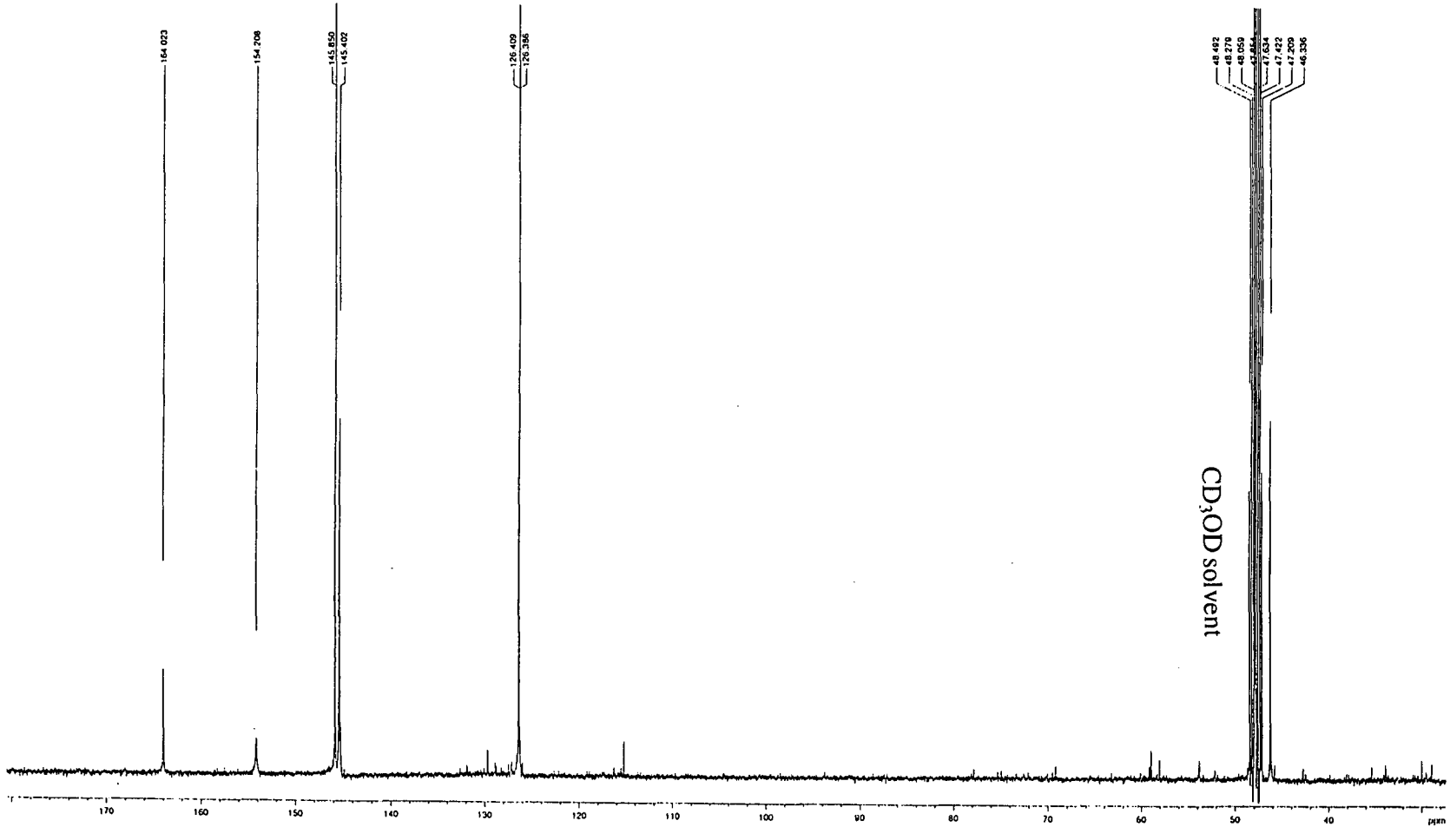


Figure 4.3.1.1 ^{13}C NMR spectrum of homarine (4.1.6.1).

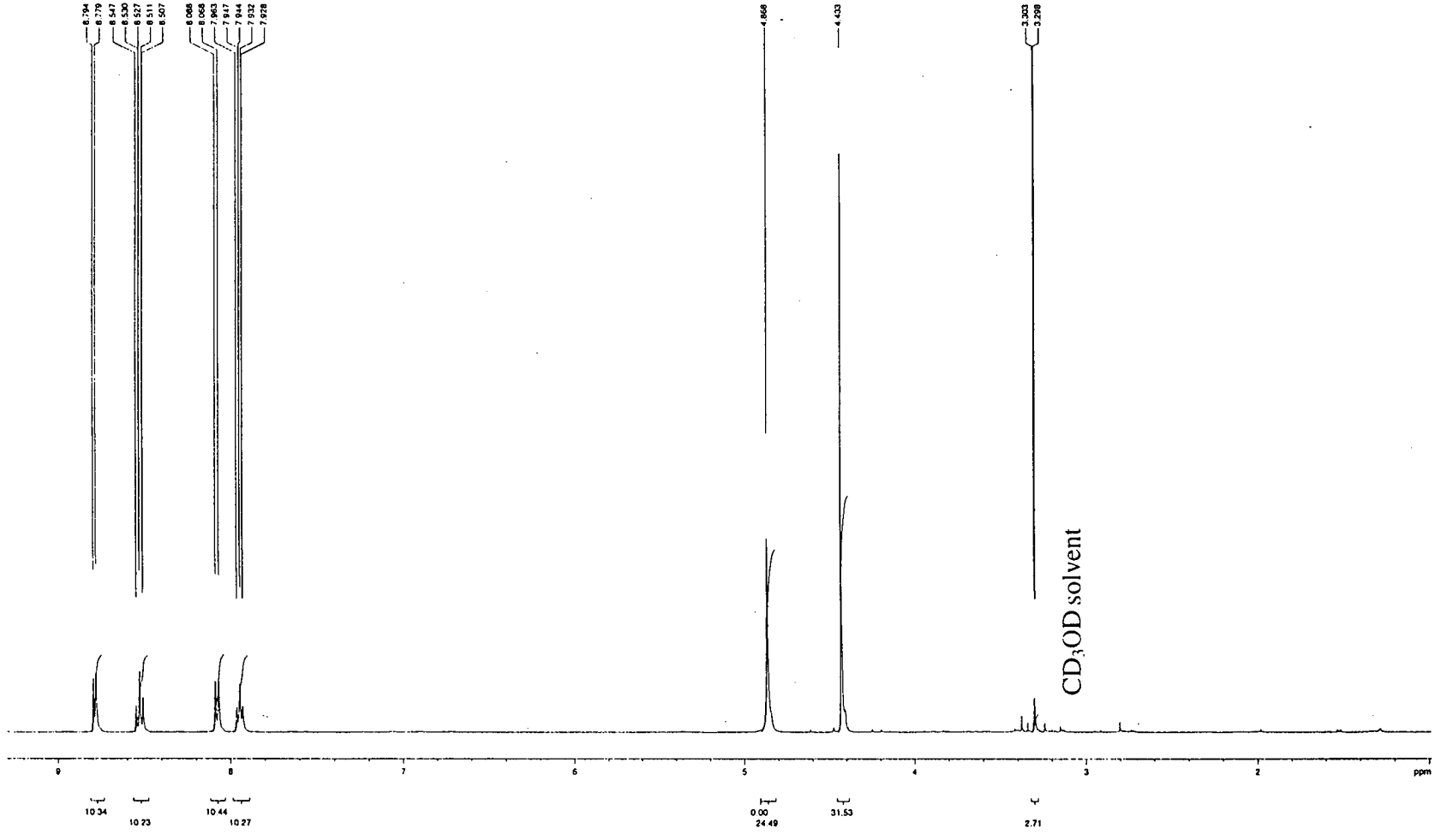


Figure 4.3.1.2 ^{1}H NMR spectrum of homarine (4.1.6.1).

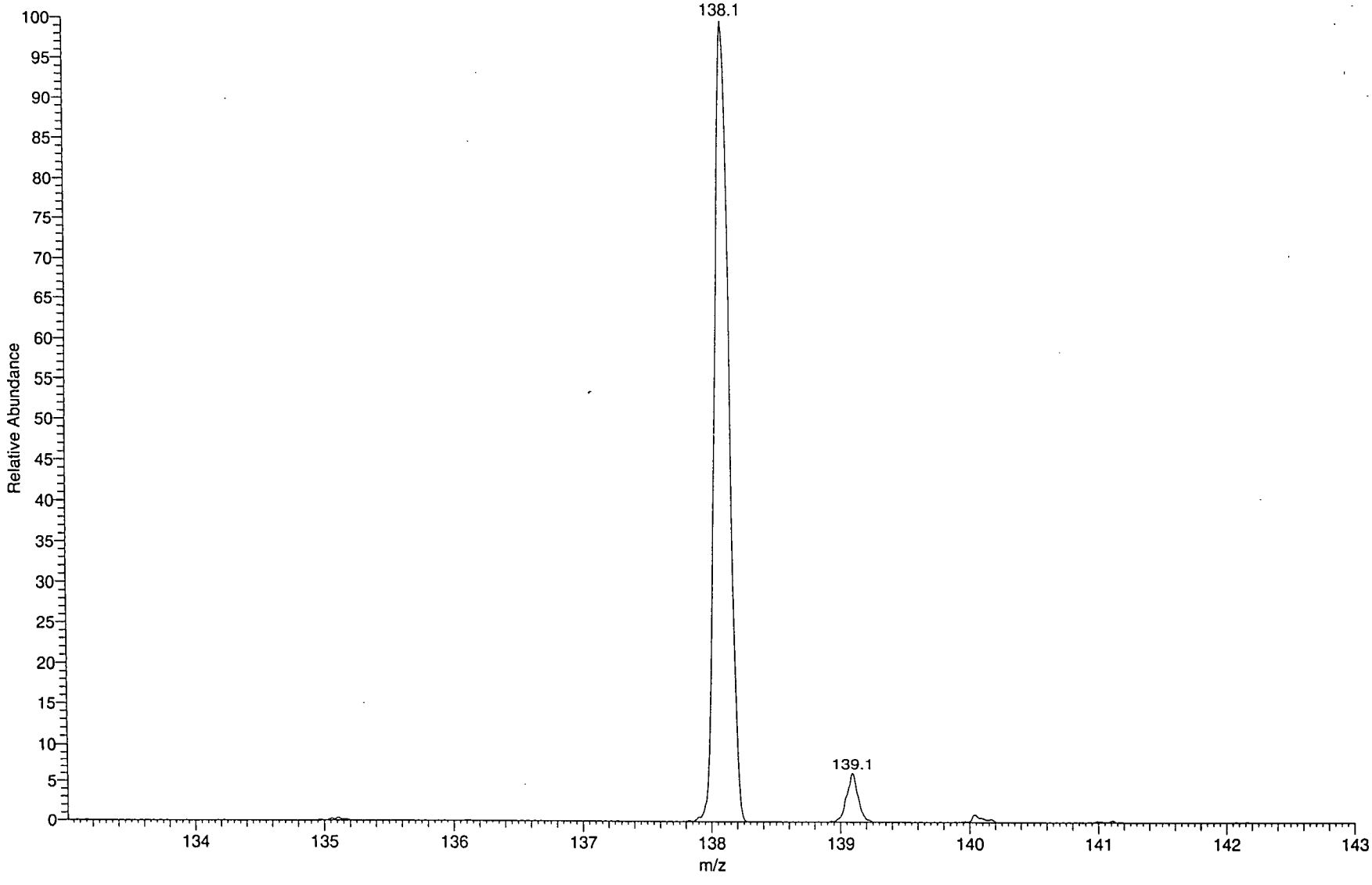


Figure 4.3.1.3 ESI LCMS data of homarine (4.1.6.1).

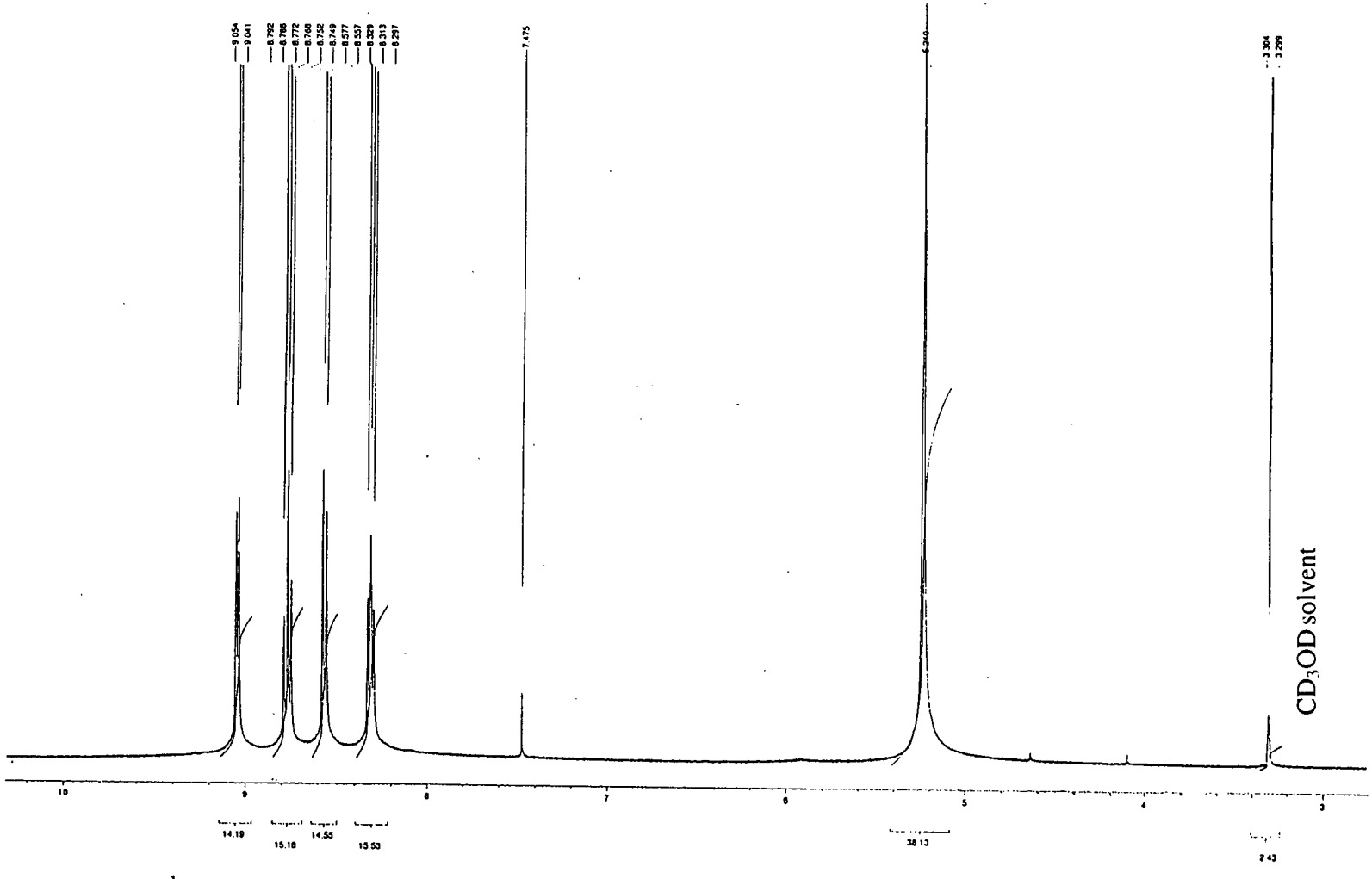


Figure 4.3.1.4 ^{1}H NMR spectrum of synthetic homarine.

Conclusion.

Homarine was a known secondary metabolite, which was isolated as a major compound from the ascidian *Polyandrocarpa lapidosa* and numerous other sources. However, time was insufficient to complete the identification of other metabolites. This is an interesting project to carry on and it is written down here and already organised for anyone who might be interested in a further study on this species, which has not yet been reported.

4.5 Experimental.

4.5.1 Collection.

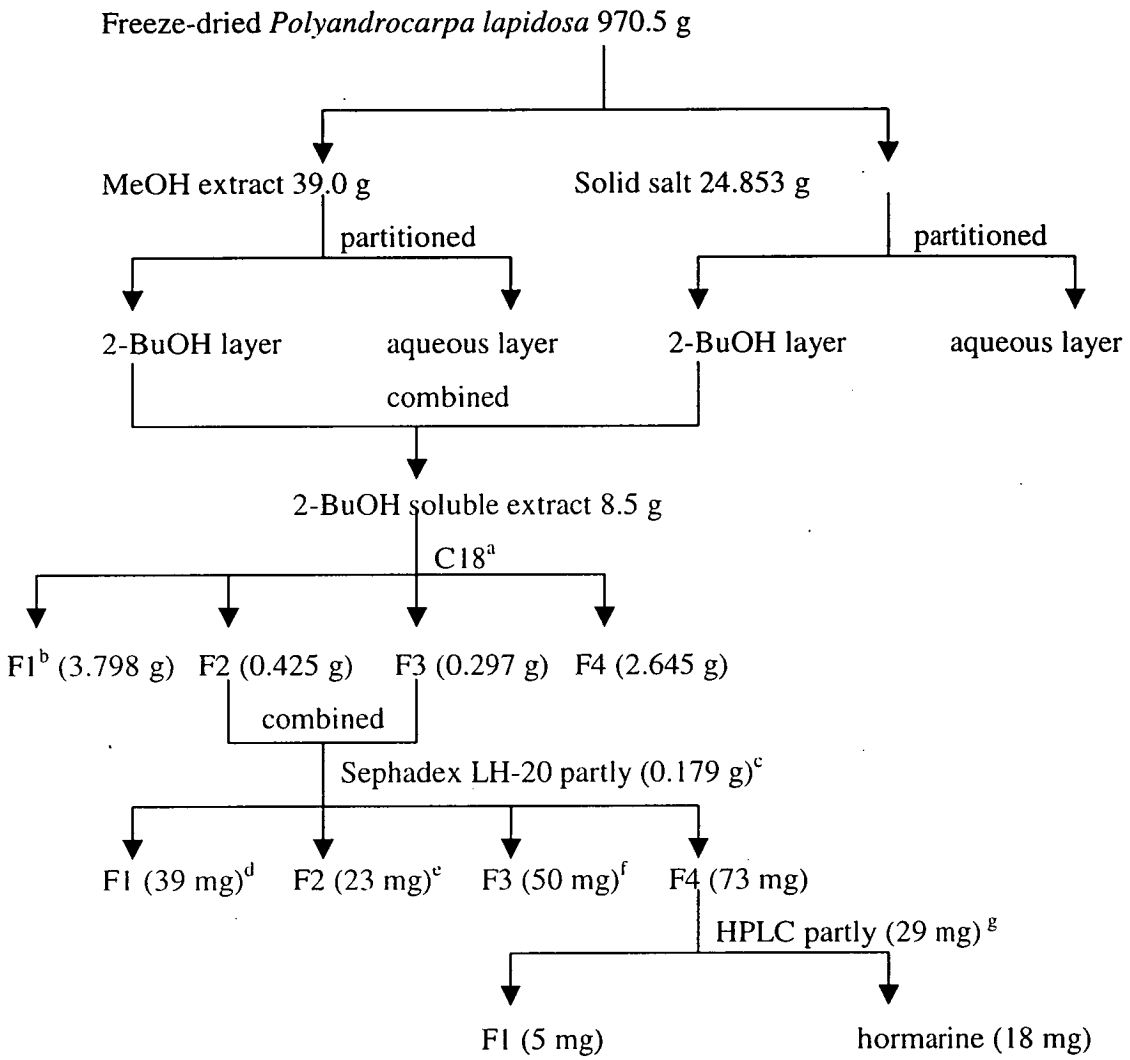
The ascidian *Polyandrocarpa lapidosa* was collected by scuba diving at a depth around 10 m off Spikey Bridge, East Coast, Tasmania on 19th Nov. 1997.

4.5.2 Extraction procedure.

The ascidian *Polyandrocarpa lapidosa* was frozen and freeze-dried. The dried sample (970.5 g) was ground with a mortar and pestle and extracted with methanol. When using methanol to extract the dried ascidian, there were a methanol soluble fraction and a solid salt residue. The solid salt (24.853 g) was filtered out. The methanol solution was concentrated on a rotary evaporator at a temperature below 30 °C to give a brown orange tar (39.0 g). The extraction was done till negative to Meyer's test. The dry residue of the ground ascidian (877.6 g) after extraction was discarded.

The methanol extract was partitioned between 2-butanol and water. The solid salt was also partitioned between 2-butanol and water. The butanol soluble material was combined and concentrated to give a brown orange tar (8.5 g), which showed positive to Meyer's test. The aqueous layer was combined and showed negative test to Meyer's reagent.

4.5.3 Separation procedure.



[a]C18 column eluting with 10-20%, 30-40%, 50-70% MeOH-H_2O and 100% MeOH.

[b]Sephadex LH-20 in MeOH to give the unidentified ionic compounds.

[c]Sephadex LH-20 in MeOH and DCM-MeOH (1:1).

[d]Contained the unidentified ionic compounds.

[e]Contained the unidentified alkaloid whose molecular weight of 352 or 541 or higher.

[f]Contained the unidentified alkaloid whose molecular weight of 174.

[g]HPLC several times in 10% MeOH-H_2O.

Scheme 4.5.3.1 Separation procedure for the ascidian *Polyandrocarpa lapidosa*.

4.5.4 Characterization of homarine.

Homarine (4.1.6.1) was isolated as transparent hydroscopic needle crystals. For the ^{13}C and the ^{1}H NMR data, see Table 4.3.1.1. The ^{1}H NMR data agreed with the synthetic homarine from piccolinic acid and methyl iodide.

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Chapter 5. Bryozoans.

5.1 General introduction and secondary metabolites from bryozoans.

The bryozoans *Watersipora subtorquata* and *Bugula dentata* (together with its associated nudibranch *Tambja verconis*), and *Cribricellina rufa* (together with its associated pycnogonid *Pseudopallene ambigua*) have been investigated in this study.

The three bryozoans are in phylum Bryozoa, class Gymnolaemata and order Cheilostomida. The bryozoan, *Watersipora subtorquata* (d' Orbigmy)[1] belongs to family Watersiporidae. It was described as an encrusting colony, with brittle and broad deep-orange margins. The bryozoan *Bugula dentata* (Lamouroux)[2] belongs to family Bugilidae. It is distributed widely and bluish-green in colour. The bryozoan *Cribricellina rufa* (MacGillivray)[3] belongs to family Catenicellidae.

Christophersen[4,5] reported secondary metabolites from bryozoans from the orders Ctenostomata and Cheilostomata. Two species of the bryozoans *Alcyonidium gelatinosum* and *Zoobotryon verticillatum* from the order Ctenostomata were reported. The bryozoan *Alcyonidium gelatinosum* yielded (2-hydroxyethyl)dimethylsulfoxonium ion. While 2,5,6-tribromo-N-methylgramine, which inhibited cell division in the fertilized sea urchin egg at ED_{50} of 16 μg/mL, was isolated from *Zoobotryon verticillatum*. Five species of the bryozoans *Bugula neritina*, *Flustra foliacea*, *Chartella papyracea*, *Sessibugula translucens* and *Phidolopora pacifica* from the order Cheilostomata were reviewed. Bryostatins, which exhibited high levels of antineoplastic activity, were isolated from *Bugula neritina*. *Flustra foliacea* gave two bromoindole alkaloids and a brominated quinoline. The two bromoindole alkaloids, flustramines A and B exhibited muscle relaxant activity *in vivo* and *in vitro* affecting both skeletal and smooth muscle. In addition, bromoindole alkaloids were obtained from *Chartella papyracea*. *Sessibugula translucens* afforded active bipyrroles. Bipyrroles, tambjamines A and B inhibited cell division at 1 μg/mL in the fertilized sea urchin egg assay and showed moderate antimicrobial activity at 50 μg/disc against *Eschericia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Vibrio anguillarum*. Tambjamines C and D inhibited cell division and showed antimicrobial activity at 5 μg/disc against *Candida albicans*, *Bacillus subtilis*, *Staphylococcus aureus* and *Vibrio anguillarum* and mild activity at 50 μg/disc against *Eschericia coli*. The bryozoan *Phidolopora pacifica* yielded an active xanthine derivative, phidolopin. Phidolopin exhibited *in vitro* antifungal activity against *Pythiam ultimum*, *Rhizoctonia solani* and *Helminthosporium sativum* with a

minimum inhibitory concentration of 70 μg/disc and antialgal activity against the diatom *Cylindrotheca fusiformis*.[4]

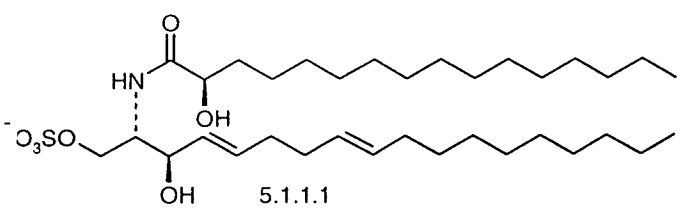
Bryozoan secondary metabolites have been reviewed by Anthoni *et al.* in 1990.[6] Microorganisms and bryozoan hosts as well as secondary metabolites were discussed. Four possibilities for the origin of the natural products isolated by bryozoans are widely known. They could be (i) true secondary metabolites of the animals, (ii) ingested and sequestered by the animal, (iii) transformed ingested compounds or (iv) derived from symbionts as such or in slightly altered form. In this paper, a hypothesis was proposed that some of the secondary metabolites isolated from bryozoans were synthesised by associated microorganisms. Bryostatins were isolated from the bryozoan *Bugula neritina*, the bryozoan *Amathia convoluta*, the sponge *Lissodendoryx isodictyalis* and the tunicate *Aplidium californicum*. Bryostatin 8 was believed to originate from the bryozoan *Amathia convoluta* while bryostatins 4, 5 and 6 were attributed the epizoic *Bugula neritina*. Bryostatins A and B were only known from the sponge and might originate from some other bryostatins partly metabolised by the sponge. Bryostatins 4 and 5 were isolated from the tunicate *Aplidium californicum*. The possibility of a dietary source of the secondary metabolites obtained from bryozoans has been implied. The bryozoan *Phidolopora pacifica* yielded phidolopin, desmethyl phidolopin and 3-nitro-4-hydroxybenzyl alcohol. One or more of these compounds were found in the taxonomically unrelated *Diaperoecia californica*, *Heteropora alaskensis*, *Tricellaria ternata* and *Hippodiplosia insculpta*. These metabolites might originate with dietary or symbiotic microorganisms. The nitro-containing secondary metabolites are known from fungi and 3,5-dinitroguaiacol has been isolated from the red alga *Marginisporum aberrans* in a low yield suggesting a possible dietary microbial origin. Although no structure closely related to the bryostatins is known, the aplasmomycins from a strain of the marine actinomycete *Streptomyces griseus* are chemically related. Another example, a blue tetrapyrrole pigment was isolated from the bryozoan *Bugula dentata*. The blue pigment was previously described from an Australian ascidian and from a mutant strain of the bacterium *Serratia marcescens*. The bacterium *S. marcescens* was possibly associated with the bryozoan and the asicidian. Similarly, Tambjamines were isolated from a green bryozoan *Sessibugula translucens*. They were considered to be precursors of the green pigment from the bryozoan, a tetrapyrrole. It was hypothesised that the tambjamines originate from the bipyrrole fragment of dietary prodigiosin known from marine bacteria of the genus *Beneckea*.

Blackman *et al.*[7] reviewed secondary metabolites from bryozoans and their chemical ecology. The secondary metabolites were classified into two major groups as non-alkaloids (macrocyclic lactones; sterols, terpenes and fatty acid derivatives; halogen- and sulfur-containing compounds), and alkaloids and related compounds (β-phenylethylamine-related alkaloids; indole alkaloids, pyrrole alkaloids; pyridine, purine, isoquinoline and β-carboline alkaloids; miscellaneous nitrogen-containing compounds).

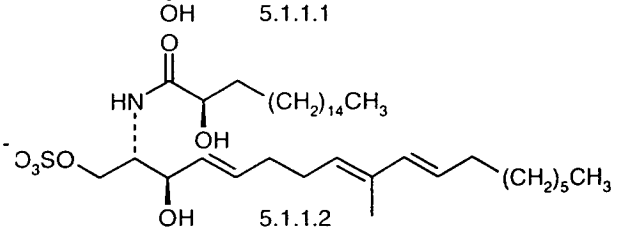
This review will cover typical secondary metabolites reported from the bryozoans genera *Watersipora*, *Bugula* as well as *Cribricellina* and the related order Cheilostomata during the period 1986 to 2000 using SciFinder Scholar. Methods of isolation, structure elucidation and biological activity will be the focus where possible. All structures will be drawn the same as in the original publications where ever possible. The order of these bryozoans genera *Watersipora*, *Bugula* and *Cribricellina* is Cheilostomata.

5.1.1 Secondary metabolites from the bryozoan *Watersipora*.

Two new potent inhibitors of a human DNA topoisomerase I, ceramide 1-sulfates (5.1.1.1-5.1.1.2) were isolated from the Japanese bryozoan *Watersipora cucullata*. The animal was collected in Aichi Prefecture, Japan. The methanol extract was partitioned to afford ethyl acetate, butanol and aqueous fractions. The butanol fraction was further purified by Sephadex LH-20, Si gel and reverse phase HPLC to give the metabolites (5.1.1.1-5.1.1.2).[8]



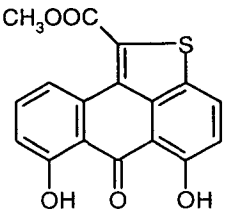
5.1.1.1



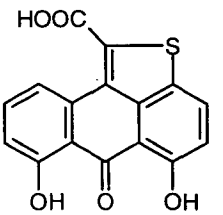
5.1.1.2

A four-step synthesis of an orange anthrathiophene pigment, 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (5.1.1.3), which was isolated from the bryozoan *Dakaira subovoidea*,[9] was described from naphthazarin.[10] The carboxylic acid form of the previous compound (5.1.1.3), namely 5,7-dihydroxy-6-oxo-6*H*-anthra[1,9-

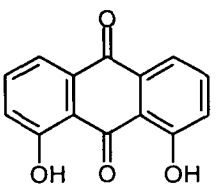
bc]thiophene-1-carboxylic acid (5.1.1.4) was found in the New Zealand bryozoan *Watersipora subtorquata*, together with another known compound, 1,8-dihydroxyanthraquinone (5.1.1.5).[11]



5.1.1.3



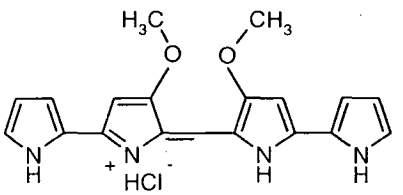
5.1.1.4



5.1.1.5

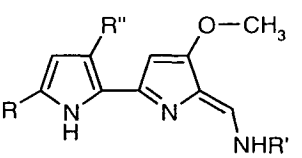
5.1.2 Secondary metabolites from the bryozoan *Bugula*.

A blue pigment (5.1.2.1) was isolated from the bryozoan *Bugula dentata*, which was collected in the Gulf of Sagami, Japan. The ethanol extract was partitioned between water and diethyl ether. The ether soluble material was chromatographed on a Si gel column with benzene-ethyl acetate (85:15) and then on a Sephadex LH-20 column with hexane-chloroform-methanol (2:1:1) to give the blue pigment (5.1.2.1).[12]



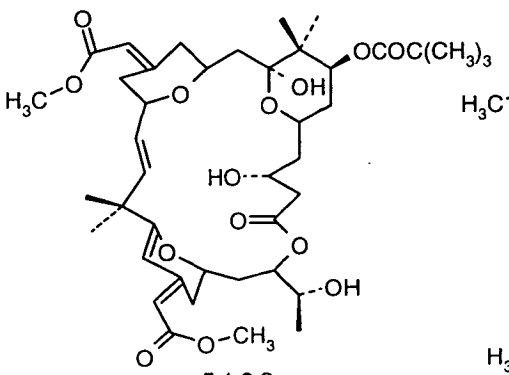
5.1.2.1

Four new alkaloids, tambjamines G (5.1.2.2), H (5.1.2.3), I (5.1.2.4), J (5.1.2.5) and two known alkaloids, tambjamines C (5.1.2.6) and E (5.1.2.7) were isolated from the bryozoan *Bugula dentata*, which was collected from the ferry terminal jetty, Kettering, Tasmania and at the North Forest Products wharf, Triabunna, Tasmania. The dichloromethane extract was purified by Si gel flash chromatography and followed by PTLC on Si gel which was impregnated with 12% sodium acetate.[13]

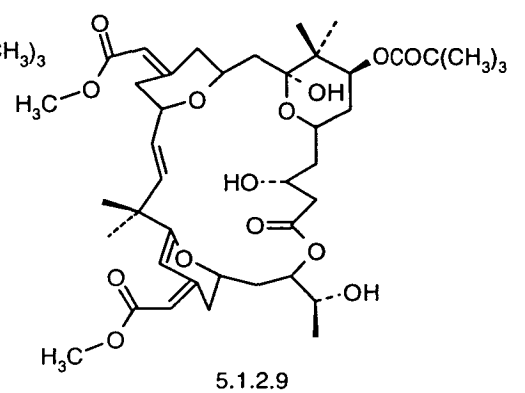


5.1.2.2 R=Br, R'=CH_2CH_3, R"=H
5.1.2.3 R=Br, R'=$CH_2CH_2CH_3$, R"=H
5.1.2.4 R=Br, R'=$CH_2CH(CH_3)_2$, R"=H
5.1.2.5 R=Br, R'=$CH_2CH(CH_3)CH_2CH_3$, R"=H
5.1.2.6 R=H, R'=$CH_2CH(CH_3)_2$, R"=H
5.1.2.7 R=H, R'=CH_2CH_3, R"=Br

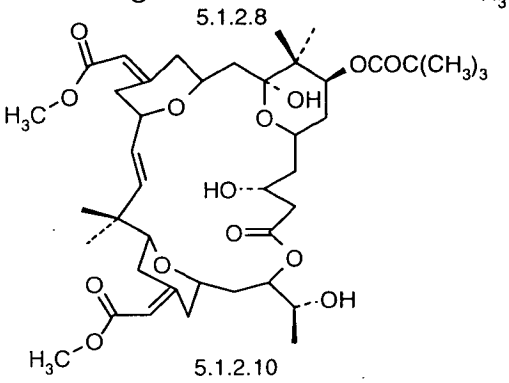
Three new 20-desoxybryostatins 16 (5.1.2.8), 17 (5.1.2.9) and 18 (5.1.2.10) were isolated from *Bugula neritina*, which was collected in the Northern Gulf of Mexico, Florida. All of the three metabolites (5.1.2.8-5.1.2.10) showed significant growth inhibitory activity against murine P388 lymphocytic leukemia.[14]



5.1.2.8



5.1.2.9

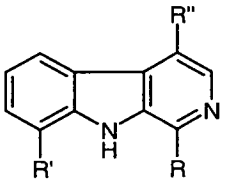


5.1.2.10

5.1.3 Secondary metabolites from the bryozoan *Cribricellina* and the related order Cheilostomata.

Harman (5.1.3.1), 1-ethyl-β-carboline (5.1.3.2), (*S*)-1-(1′-hydroxyethyl-β-carboline) (5.1.3.3), and pavettine (5.1.3.4) were isolated from the bryozoan *Costaticella hastata*, which was collected near the Blowhole, Eaglehawk Neck, Tasman Peninsula and from Tinderbox, River Derwent Estuary, near Hobart, Tasmania.[15]

Two new cytotoxic β-carboline alkaloids, 1-vinyl-8-hydroxy-β-carboline (5.1.3.5) and 1-ethyl-4-methylsulfone-β-carboline (5.1.3.6), together with three known compounds, harman (5.1.3.1), 1-ethyl-β-carboline (5.1.3.2), and pavettine (5.1.3.4) were isolated from the bryozoan *Cribricellina cribraria*. The animal was collected from Sugar Loaf, Kaikoura, off the South Island of New Zealand. The methanol-toluene extract was purified by reverse phase C18 several times and Si gel TLC to give the metabolites (5.1.3.5-5.1.3.6).[16]



5.1.3.1 R=CH_3, R'=R"=H
5.1.3.2 R=Et, R'=R"=H
5.1.3.3 R=$CH(OH)CH_3$, R'=R"=H
5.1.3.4 R=$CH=CH_2$, R'=R"=H
5.1.3.5 R=$CH=CH_2$, R'=OH, R"=H
5.1.3.6 R=Et, R'=H, R"=$S(O)_2CH_3$

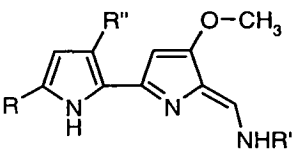
5.2 General introduction and secondary metabolites from nudibranchs.

The nudibranch *Tambja verconis* (Basedow & Hedley, 1905) belongs to phylum Mollusca, class Gastropoda, subclass Opisthobranchia, order Nudibranchia, and family Polyceridae. This nudibranch is distinctive, has a yellow body with sky-blue markings and is apparently present at all locations where the bryozoan *Bugula dentata* occurs.[17]

The defensive roles of secondary metabolites from sponges and nudibranchs have been reviewed.[18] Novel compounds from all three subclasses of the phylum Mollusca, namely Prosobranchia, Opisthobranchia and Pulmonata, were reviewed.[19] Natural products of opisthobranch mollusks, both in terms of an origin and activity including localization of the metabolites, as well as contribution to taxonomy and ecology have been reported.[20] Chemistry and ecology of marine opisthobranch molluscs, order Sacoglossa and Nudibranchia were summarized.[21] Diterpenes from marine opisthobranch molluscs, particularly bioactive ones, has been reviewed.[22]

This review will describe typical secondary metabolites from the nudibranch genus *Tambja* during the period 1983 to 2000 using SciFinder Scholar. The method of isolation, structure elucidation, and biological activity will be the focus where possible.

Carté and Faulkner isolated tambjamines A (5.2.1), B (5.2.2), C (5.1.2.6) and D (5.2.3) from three nembrothid nudibranchs *Tambja abdere*, *Tambja eliora*, and *Roboastra tigris*, which were collected at Puerto Escondido, Baja California, Mexico, at Bahia de los Angeles, Baja California, and at Isla Partida, Gulf of California.[23] The role of the secondary metabolites, tambjamines A-D in feeding associations between the nudibranchs (*Tambja abdere*, *Tambja eliora*, and *Roboastra tigris*) and the bryozoan *Sessibugula translucens* was studied.[24]



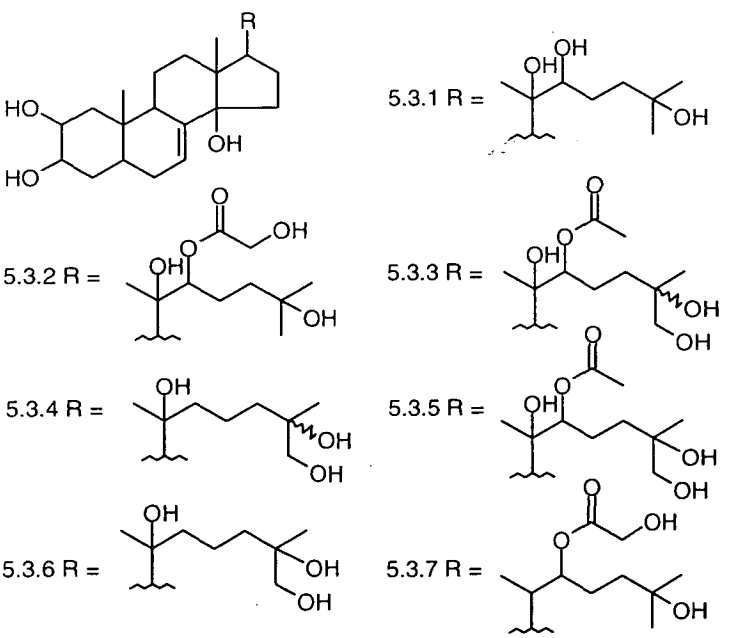
5.2.1 R=R'=R"=H
5.2.2 R=Br, R'=R"=H
5.2.3 R=H, R'=$CH_2CH(CH_3)_2$, R"=Br

5.3 General introduction and secondary metabolites from pycnogonids.

The associated pycnogonid *Pseudopallene ambigua* belongs to phylum Arthopoda, subphylum Chelicerata, class Pycnogonida, and family Callipallenidae.[25] It is recorded in association with bryozoans.

This review will describe typical secondary metabolites from pycnogonids during the period 1983 to 2000 using SciFinder Scholar. The method of isolation, structure elucidation, and biological activity will be the focus where possible.

A study of defensive secretion of ecdysteroids (5.3.1-5.3.8) from the pycnogonid *Pycnogonum litorale* showed a significant feeding deterrence of its predator, the common shore crab *Carcinus maenas*. The arthopod molting hormone, 20-hydroxyecdysone and the predominant ecdysteroid in the pycnogonid, 20-hydroxyecdysone 22-acetate, were tested for the antifeeding effect on *Carcinus maenas*.[26, 27]



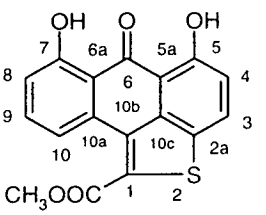
5.4 Results and discussion.

5.4.1 Secondary metabolites from the bryozoan *Watersipora subtorquata*.

The bryozoan *Watersipora subtorquata* was collected from Triabunna, Tasmania by scuba diving. Freeze-dried *Watersipora subtorquata* was extracted with methanol-dichloromethane (3:1). Ethyl acetate was used to dissolve the methanol-dichloromethane crude extract. The ethyl acetate soluble fraction was further purified by Si gel open-column, Si gel MPLC column, C18 column twice and then Sephadex LH-20 column to afford the known compounds (5.1.1.3 and 5.1.1.5).

5.4.1.1 The structure of 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (5.1.1.3).

The APCI LCMS gave $[M+H]^+$ 327.3 showing intensities of the ion peaks which suggested the presence of the [A+2] atom, sulfur in the molecule (Figure A5.4.1.1.1), corresponding to the molecular weight of 326 of $C_{17}H_{10}O_5S$. The ^1H NMR data (Table 5.4.1.1.1) was compared with the literature data.[9]



5.1.1.3

Table 5.4.1.1.1 ^1H NMR data of the compound (5.1.1.3) and from the literature.[9]

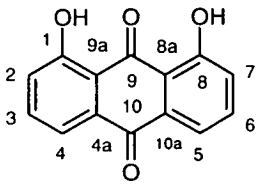
No.	$^1H^a$, *J* (Hz) of (5.1.1.3)	$^1H^b$, *J* (Hz) from literature[9]
1b	4.59 s	4.05 s
3	7.59 d, 8.4	7.28 d, 8.82
4	6.82 d, 8.4, 23.2	7.12 dd, 1.0, 8.3
8	6.82 d, 8.4, 23.2	8.88 dd, 1.0, 8.3
9	7.48 t	7.68 t, 8.3
10	9.54 d	8.02 d, 8.8

[a] recorded at 400 MHz, using CD_3OD as a solvent.

[b] recorded at 270 MHz, using $CDCl_3$ as a solvent, also shown 12.35 s and 13.21 s.

5.4.1.2 The structure of 1,8-dihydroxyanthraquinone (5.1.1.5).

^{1}H NMR data (Table 5.4.1.2.1) of the compound (5.1.1.5) was consistent with the data from the literature for a New Zealand sample.[11]



5.1.1.5

Table 5.4.1.2.1 ^{1}H NMR data of the compound (5.1.1.5), $CDCl_3$ solvent.

No.	^{1}H[a] of (5.1.1.5)	^{1}H[b], *J* (Hz) of (5.1.1.5)[11]
1-OH	11.00 s	12.06 s
2	7.30 d	7.30 dd, 8.4, 1.1
3	7.50 t	7.68 t, 8.1
4	7.85 d	7.84 dd, 7.5, 1.1
5	7.85 d	7.84 dd, 7.5, 1.1
6	7.50 t	7.68 t, 8.1
7	7.30 d	7.30 dd, 8.4, 1.1
8-OH	11.00 s	12.06 s

[a] recorded at 200 MHz.

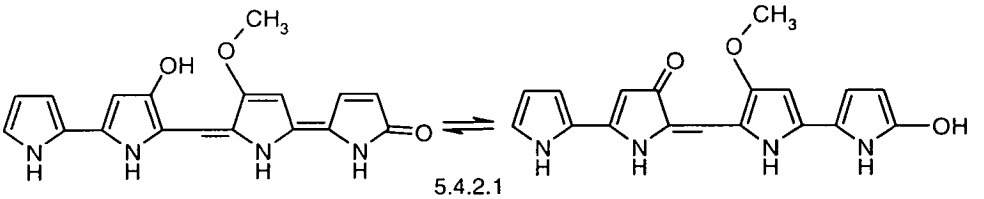
[b] recorded at 300 MHz.

5.4.2 Secondary metabolites from the bryozoan *Bugula dentata*.

The bryozoan *Bugula dentata* (39.782 g wet weight) was collected from Triabunna, Tasmania by scuba diving. *Bugula dentata* was extracted with methanol-dichloromethane (1:1) to give a viscous crude extract (5.60% yield base on the bryozoan wet weight). The extract was further purified by Si gel open-column, and preparative TLC to afford tambjamines A (5.2.1), B (5.2.2), C (5.1.2.6), D (5.2.3), E (5.1.2.7), G (5.1.2.2), H (5.1.2.3), and J (5.1.2.5), together with some unidentified compounds (molecular weight 336, 259, 361 and 439/441 with Br pattern). Tambjamines A (5.2.1) and G (5.1.2.2) were isolated in pure form.

Interestingly, a blue pigment (0.020% yield base on the animal wet weight) was isolated from the Sephadex LH-20 column when eluted with methanol-dichloromethane (50:50) and showed molecular weight of 336 by LSIMS technique (as well as ESI LCMS

experiment), giving $C_{18}H_{17}N_4O_3$ $[M+H]^+$ obs. 337.12818 and cal. 337.13007, which corresponds to thirteen degrees of unsaturation. It is notable that the known blue pigment previously isolated from the same species[12] has a molecular weight of 334 corresponding to the molecular formula $C_{19}H_{18}N_4O_2$. In this study the blue pigment has one carbon and two hydrogens less but one oxygen more than the previous blue pigment (5.1.2.1). The ^1H spectrum (Figure 5.4.2.1) displayed a methoxy group at 3.95 ppm and the ^{13}C NMR spectrum (Figure 5.4.2.2) showed carbonyl carbons at 177.3 and 177.1 ppm. Therefore, it is possible that the blue pigment in this study might be eg. compound (5.4.2.1) which could exist as a tautomeric mixture. The MS/MS data showed 337 (M+H)→322 (M+H-15)→307 (M+H-15-15) and 322 (M+H-15)→304 (M+H-15-18)→276 (M+H-15-18-28)→248 (M+H-15-18-28-28). The structure (5.4.2.1) does not seem to be consistent with the MS/MS data, which had two consecutive losses of 15. This suggested that a further study is needed.



5.4.2.1

The next unidentified compounds were obtained as a mixture of an unbrominated compound (molecular weight 361) and a mono brominated compound (molecular weight 439) by LCMS through C18 analytical column, methanol-water (80:20) with 0.1% ammonium acetate isocratic condition for 4 minutes then gradient to methanol-water (95:5) with 0.1% ammonium acetate. For the unbrominated compound, the EI MS (peak match measurement) showed $C_{20}H_{19}N_5O_2$ $[M]^+$ obs. 361.14996 and cal. 361.15137, The MS/MS data showed 362 (M+H)→345 (M+H-17)→330 (M+H-17-15)→315→298→267 and 345→314, 345→299 as well as 315→287, 315→258, 315→195 together with 298→167. For the monobrominated compound, the EI MS measurement showed $C_{20}H_{18}BrN_5O_2$ $[M]^+$ obs. 439.06340 and cal. 439.06439, corresponding to fourteen degrees of unsaturation. The MS/MS data showed 440/442 (M+H)→423/425 (M+H-17)→408/410 (M+H-17-15)→393/395 (M+H-17-15-15), 423/425→392/394 (M+H-17-31), 423/425→344 (M+H-17-79), 423/425→377/379 (M+H-17-46), and 408/410→329 (M+H-17-15-79). The mixture's ^1H and ^{13}C NMR spectra are presented in Figure 5.4.2.3 and Figure 5.4.2.4, respectively.

The last unidentified compound was found in the fraction several times by LCMS, it showed a molecular weight of 259. Unfortunately there was insufficient time to continue with further purification and structural determination of these still unidentified compounds.

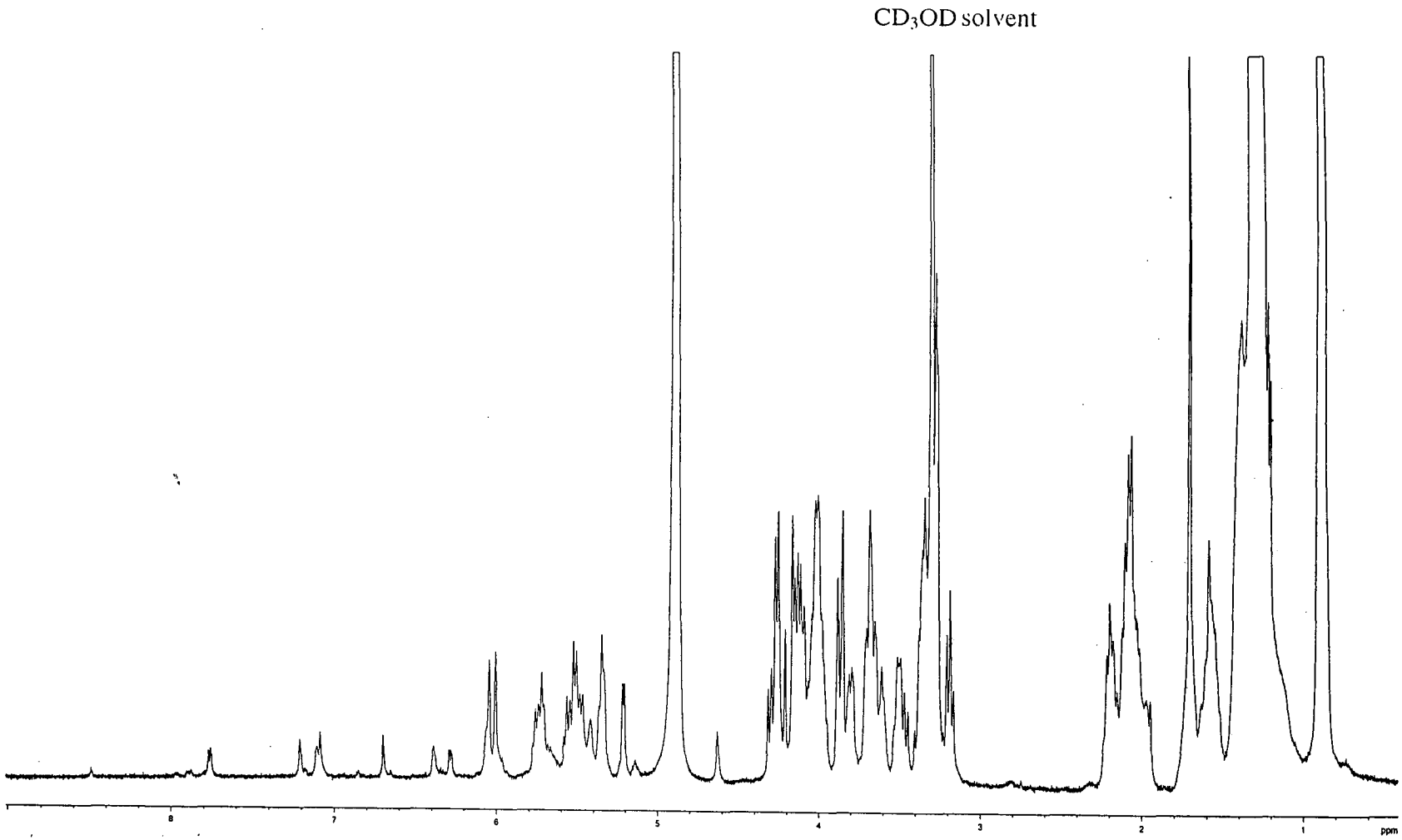


Figure 5.4.2.1 ^{1}H NMR spectrum of the blue pigment MW 336.

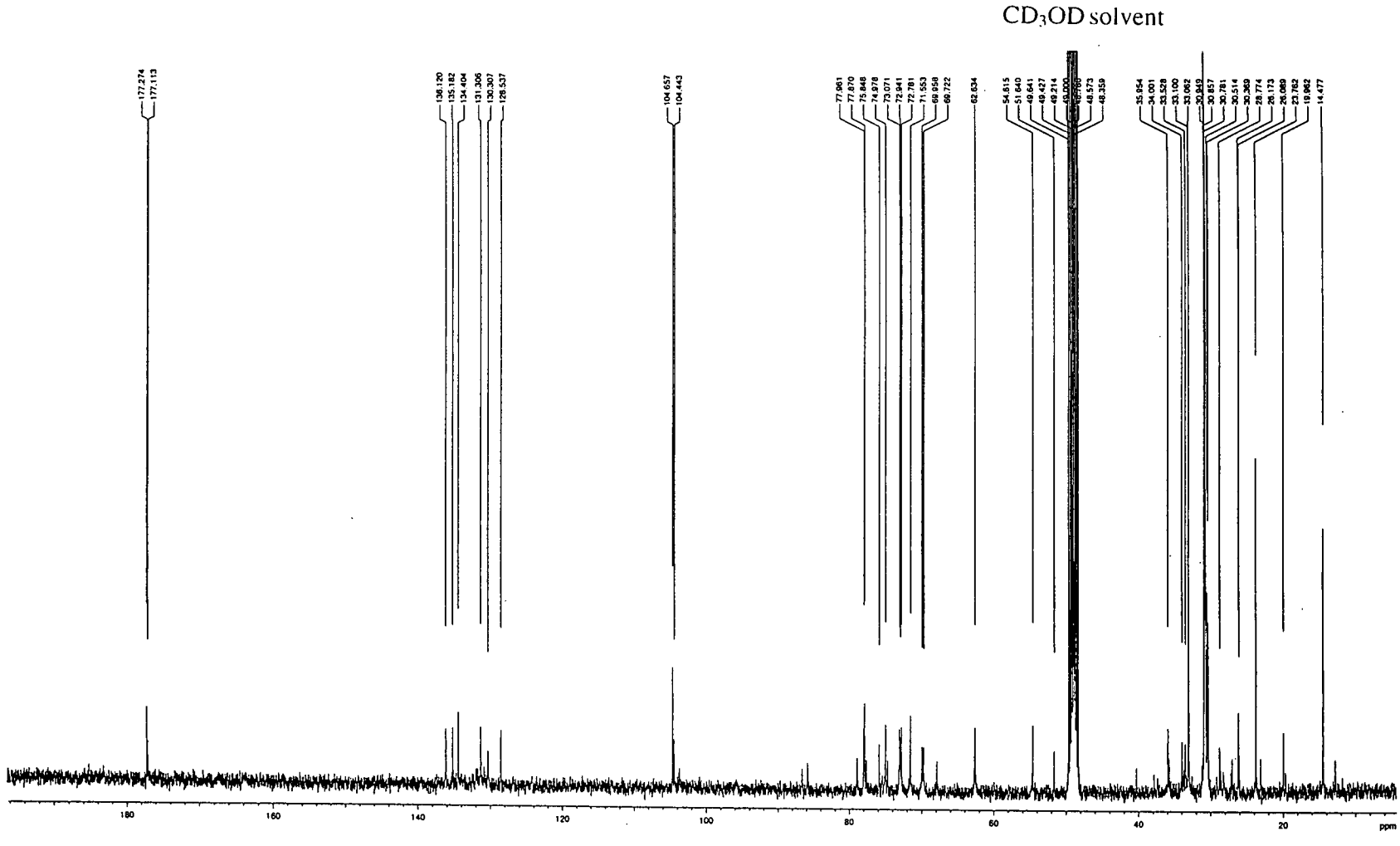


Figure 5.4.2.2 ^{13}C NMR spectrum of the blue pigment MW 336.

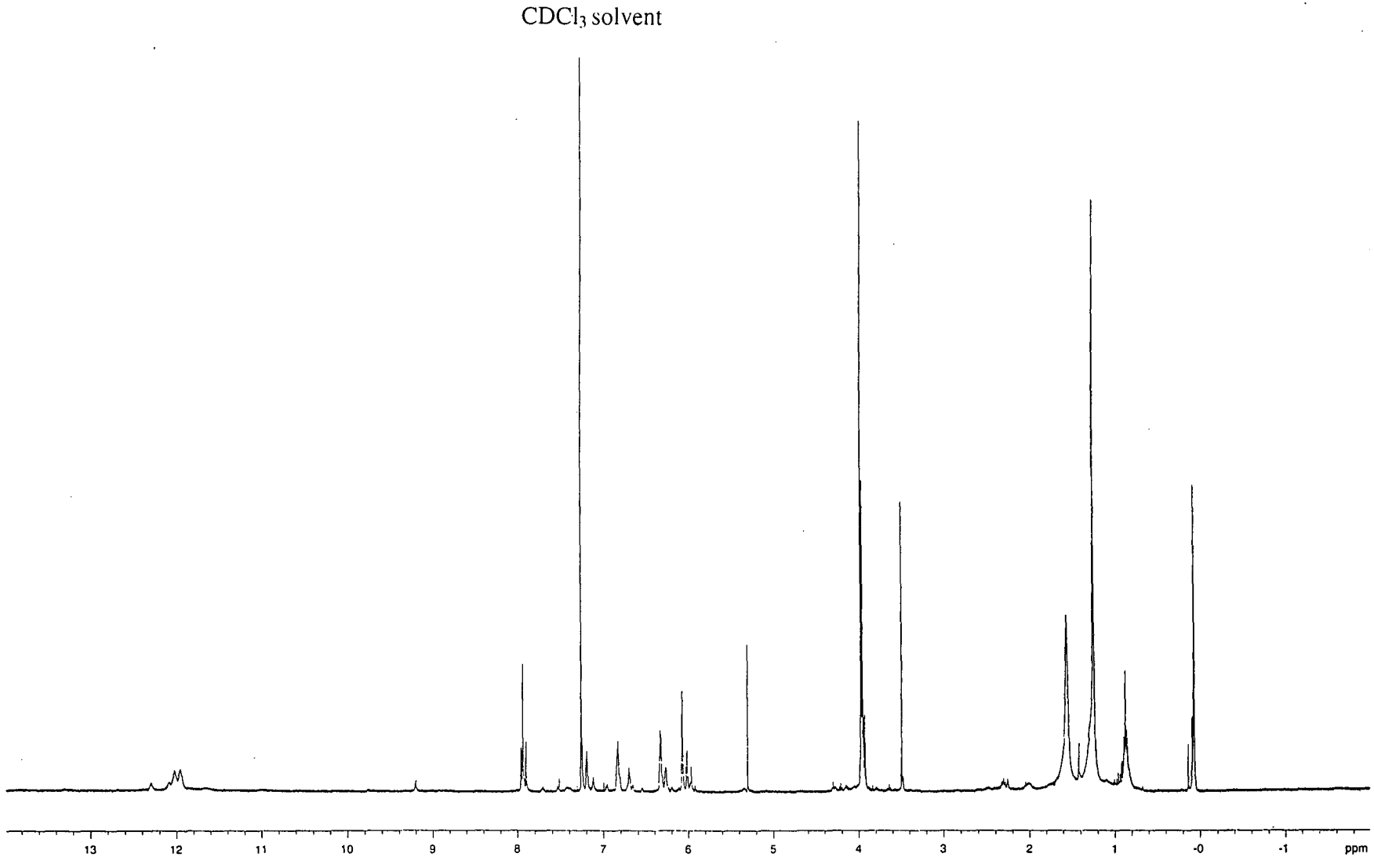


Figure 5.4.2.3 ^{1}H NMR spectrum of the mixture of compounds MW 361 and 439/441.

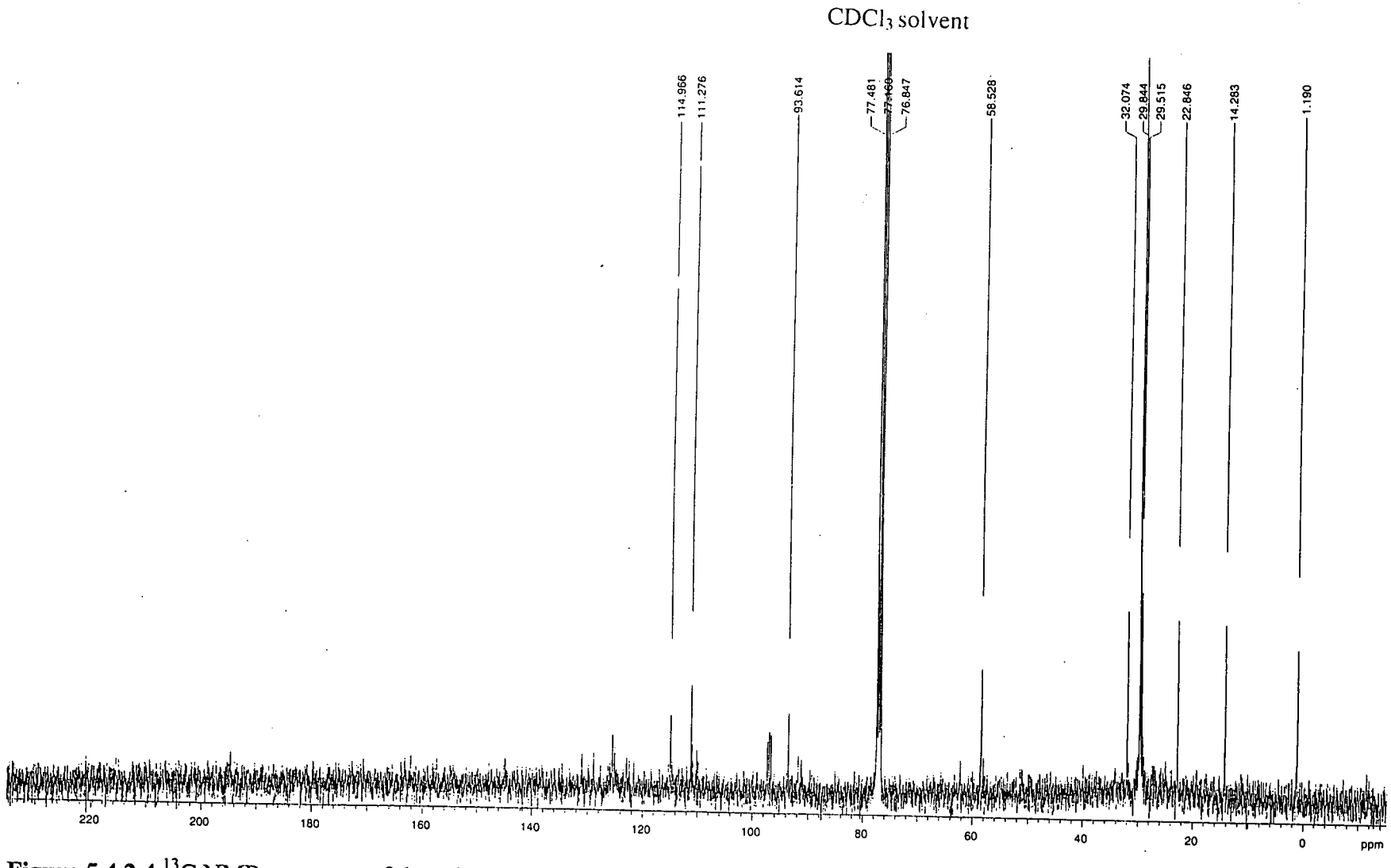


Figure 5.4.2.4 ^{13}C NMR spectrum of the mixture of compounds MW 361 and 439/441.

5.4.3 Secondary metabolites from the nudibranch *Tambja verconis*.

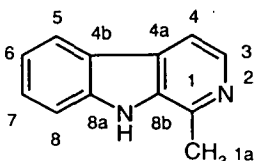
The nudibranch *Tambja verconis* was extracted with dichloromethane and the extract was purified through Si gel open-column. The results showed that all the compounds from the bryozoan *Bugula dentata* were also present in the nudibranch *Tambja verconis*. However, time was limited so it was not possible to separate all of the compounds found in both organisms.

5.4.4 Secondary metabolites from the bryozoan *Cribricellina rufa*.

The bryozoan *Cribricellina rufa* (32.3 g dry weight) was collected twice from the same place at Bicheno, jetty area, Tasmania by scuba diving on 27th Oct. 1998 and 6th Sept. 1999. Freeze-dried *Cribricellina rufa* was extracted with methanol-dichloromethane (1:1) to give a viscous crude extract (0.69% yield base on the bryozoan dry weight), showing a positive test to Meyer's reagent. The extract was further purified using Si gel open-column and preparative TLC to afford seven β-carboline derivatives (harman and compound nos. 1-6 by molecular weight ordering). Although not all of these compounds, nor those of the associated pycnogonid *Pseudopallene ambigua*, have been identified because of insufficient time, the results are documented here because this preliminary investigation indicates several potentially novel components and interesting chemical ecological relationships that should be further studied.

5.4.4.1 Harman.

Harman or 1-methyl-β-carboline (5.3.1) was isolated as 91.5% pure by GCMS. The GCMS spectrum (Figure A5.4.4.1.2) showed m/z 183 (14), 182 (M^+, 100), 181 (31), 154 (25), 140 (5), 127 (9), 115 (2), 114 (3), 113 (4). Both GCMS and ^{1}H NMR (Figure 5.4.4.1.1, Table 5.4.4.1.1) data were consistent with the literature.[28, 29, 30] Harman was isolated from some terrestrial plants such as in the family Simaroubaceae, eg. *Picrasmaja javanica*.[28] A review in 1975 was reported that harman was isolated from 23 plant species belonging to 8 families.[31] Then five years later, another review was described that harman was distributed in 45 plant species belonging to 15 families.[32] Later on it was isolated from the Tasmanian bryozoan *Costaticella hastata*,[15] as well as from the New Zealand bryozoan *Cribricellina cribraria*.[16]



5.1.3.1

Table 5.4.4.1.1 ^{1}H NMR data of harman (5.1.3.1), $CDCl_3$ solvent.

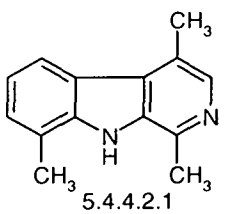
No.	$^1H^a$ of (5.1.3.1)	$^1H^b$, *J* (Hz) of (5.1.3.1)[28]
1a	2.92 s	not reported
3	8.32 br	8.37 d, 5.4
4	7.85 d, 5.0	7.81 d, 5.4
5	8.13 d, 10.0	8.1 q
6	7.30, 7.60 m	7.2-7.5 m
7	7.30, 7.60 m	7.2-7.5 m
8	7.30, 7.60 m	7.2-7.5 m

[a] recorded at 200 MHz.

[b] recorded at 100 MHz.

5.4.4.2 Compound no. 1.

The GCMS spectrum (Figure A5.4.4.2.1) of compound no. 1 showed m/z 222 (M^+, 70), 208 (21), 207 (100), 206 (47), 205 (22), 182 (18), 148 (7), 127 (7), 103 (14). Losses of 15 twice could be accounted for by two methyl groups being added to harman. Compound no. 1 could possibly be one of the isomers of dimethyl harman, for example compound (5.4.4.2.1). A common mass fragment of 182 from harman could be a reason that compound no. 1 has been related to harman.

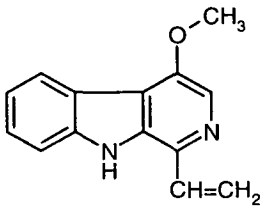


5.4.4.2.1

5.4.4.3 Compound no. 2.

Compound no. 2 was isolated as 98% pure by GCMS. This compound was the second most major component from the bryozoan *Cribricellina rufa*. The GCMS spectrum (Figure A5.4.4.3.1) of compound no. 2 showed m/z 224 (M^+, 10), 209 (9), 182 (100), 168 (4), 154 (12), 140 (3). Vinyl and methyl groups were present because of the losses of 27 and 15. However, the mass spectrum of compound no. 2 was different from the literature

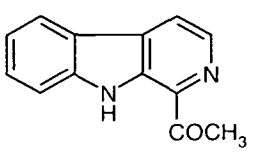
data[28, 29] of dehydrocrenatine's mass spectrum, which showed a significant of the peak 224 (M^+, 100) as a base peak. Compound no. 2 was thus an isomer of dehydrocrenatine. The structure of dehydrocrenatine or 4-methoxy-1-vinyl-β-carboline (5.4.4.3.1) itself is shown below. Dehydrocrenatine was isolated from the bark and roots of terrestrial plant *Ailanthus malabarica*, family Simarubaceae.[33]



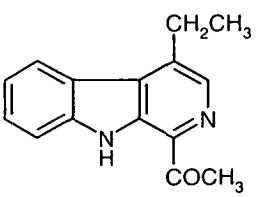
5.4.4.3.1

5.4.4.4 Compound no. 3.

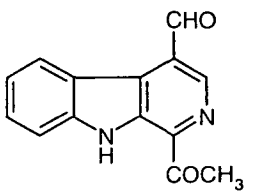
Compound no. 3 was the second major compound from this bryozoan. The GCMS spectrum (Figure A5.4.4.4.1) of compound no. 3 showed m/z 238 (M^+, 15), 223 (6), 219 (2), 210 (13), 195 (14), 182 (18), 169 (14), 168 (100), 167 (32), 166 (7), 140 (27), 114 (10). Losses of 28 and 42 were detected, which was similar to the fragmentation[30, 33] of 1-acetyl-β-carboline (5.4.4.4.1). Nevertheless, another loss of 28, that indicated either ethyl or formyl groups, was added to 1-acetyl-β-carboline. Therefore, compound no. 3 could be 1-acetyl-4-ethyl-β-carboline (5.4.4.4.2) or an isomer of it or could be 1-acetyl-4-formyl-β-carboline (5.4.4.4.3) or its isomer.



5.4.4.4.1



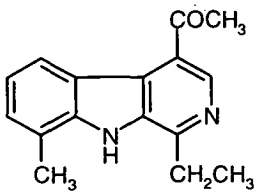
5.4.4.4.2



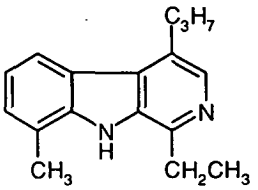
5.4.4.4.3

5.4.4.5 Compound no. 4.

Compound no. 4 was isolated as 84.5% pure by GCMS and was the third most abundant compound from this bryozoan. The GCMS spectrum (Figure A5.4.4.5.1) showed m/z 254 (M^+, 14), 224 (11), 223 (20), 213 (6), 212 (380, 211 (100), 197 (35), 182 (27), 168 (25), 140 (12) with a loss of 43, which corresponded to an acetyl group or a propyl group. Other losses of 14, 15 and 14 were accounted for by ethyl and methyl groups. Compound no. 4 was thus compound (5.4.4.5.1) or an isomer of it or compound (5.4.4.5.2) or its isomer.



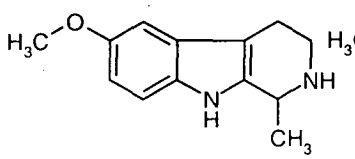
5.4.4.5.1



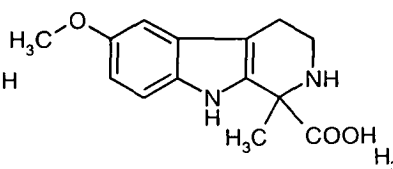
5.4.4.5.2

5.4.4.6 Compound no. 5.

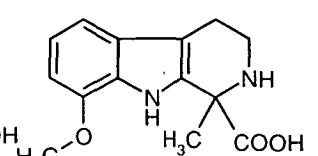
The GCMS spectrum (Figure A5.4.4.6.1) of compound no. 5 showed m/z 260 (2), 259 (3), 258 (M^+, 10), 257 (8), 243 (8), 227 (2), 219 (5), 218 (36), 217 (20), 216 (100), 215 (8), 202 (3), 181 (7). A loss of 42 between the base peak and the molecular ion peak suggested that it was either an acetyl or propyl derivative of 1,2,3,4-tetrahydro-6-methoxy-1-methyl-β-carboline or an isomer. The GCMS database[29] of 1,2,3,4-tetrahydro-6-methoxy-1-methyl-β-carboline (5.4.4.6.1) itself showed m/z 216 (M^+, 67), 201 (100), 187 (43), 172 (15), 158 (7), 144 (9), 130 (5), 115 (5). However, compound no. 5 was neither 1,2,3,4-tetrahydro-1-carboxy-6-methoxy-1-methyl-β-carboline (5.4.4.6.2) or 1,2,3,4-tetrahydro-1-carboxy-8-methoxy-1-methyl-β-carboline (5.4.4.6.3) but it might be an isomer of them as the GCMS spectrum of compound no. 5 was different from that of (5.4.4.6.2) (m/z 260 (M^+, 2), 216 (100), 245 (1), 201 (94), 160 (15), 147 (10), 132 (5))[29] and that of (5.4.4.6.3) (m/z 260 (M^+, 7), 245 (2), 216 (100), 200 (40), 186 (10), 170 (8), 160 (5), 108 (10)).[29]



5.4.4.6.1



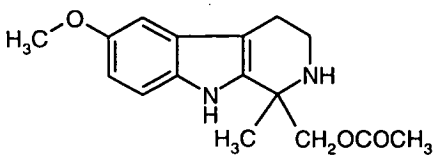
5.4.4.6.2



5.4.4.6.3

5.4.4.7 Compound no. 6.

Compound no. 6 was isolated as 99% pure by GCMS. The GCMS spectrum (Figure A5.4.4.7.1) showed m/z 288 (M^+, 9), 257 (16), 245 (100), 231 (26), 216 (21), 202 (18), 182 (7). A loss of 43 between the base peak and the molecular ion peak or a loss of 72 between the peak at 216 and the molecular ion peak suggested $-CH_2-O-CO-CH_3$ group attached to the tetrahydro-derivative (5.4.4.6.1). Therefore, compound no. 6 might be 1,2,3,4-tetrahydro-1-methylacetoxy-6-methoxy-1-methyl-β-carboline (5.4.4.7.1) or an isomer of it.



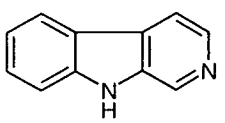
5.4.4.7.1

5.4.5 Secondary metabolites of the pycnogonid *Pseudopallene ambigua*.

The pycnogonid *Pseudopallene ambigua* was associated with the bryozoan *Cribricellina rufa* and was collected twice from the same place at Bicheno, jetty area on 27th Oct. 1998 and 6th Sept. 1999 concurrently with collections of the bryozoan. The wet sample 3.431 g of the pycnogonid was extracted with methanol-dichloromethane (1:1) to give a crude extract (8.34% yield base on the pycnogonid wet weight), showing a positive test to Meyer's reagent. The extract was further purified using Si gel open-column and preparative TLC to afford seventeen β-carboline derivatives (norharman, harman, pavettine, 1-ethyl-β-carboline, 1-acetyl-β-carboline, compound nos. 2, 3 and 7-16). Three of them (harman, compound nos. 2, and 3) were the same as the bryozoan *Cribricellina rufa*, while the others were different; not all have been identified since time was limited.

5.4.5.1 Norharman (5.4.5.1.1).

The GCMS spectrum (Figure A5.4.5.1.1) showing m/z 168 (M^+, 100), 140 (20), 114 (12), 113 (10) exactly matched with the literature data[29, 30] of norharman (5.4.5.1.1). Norharman was isolated from terrestrial plant *Chrysophyllum lacourtianum* and also occurs in tobacco smoke.[34]



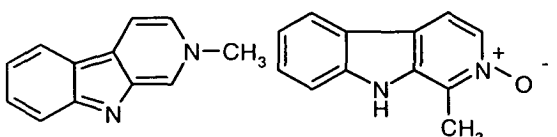
5.4.5.1.1

5.4.5.2 Harman (5.1.3.1).

Harman was isolated as 91.5% pure by GCMS from the pycnogonid *Pseudopallene ambigua*. The GCMS spectrum showing m/z 182 (M^+, 100), 154 (25), 140 (5), 127 (9) matched with the literature data[29, 30] of harman (5.1.3.1), which has also been found in the bryozoan *Cribricellina rufa* in this study.

5.4.5.3 Compound no. 7.

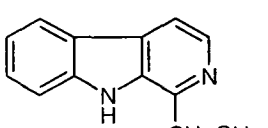
Compound no. 7 had the same molecular weight as harman although the GCMS spectrum (Figure A5.4.5.3.1) was different from that of harman, showing m/z 182 (M^+, 17), 168 (100), 154 (5), 140 (22), 128 (3), 114 (14), 113 (11). The GCMS spectrum of neither 2-methyl-β-carboline (5.4.5.3.1)[29] nor harman-2-oxide (5.4.5.3.2)[35], which did not have a molecular ion peak of 199 but had a peak of 182 (M^+-17), matched. Compound no. 7 is likely to be an isomer of harman.



5.4.5.3.1 5.4.5.3.2

5.4.5.4 Pavettine (5.1.3.4).

The GCMS spectrum (Figure A5.4.5.4.1) showed m/z 195 (12), 194 (M^+, 73), 193 (100), 168 (18), 140 (12). A loss of 27 corresponded to a vinyl group (attached to β-carboline). The mass spectrum matched with the literature data[15, 16, 36] of pavettine or 1-vinyl-β-carboline (5.1.3.4). Pavettine was isolated from the plant *Pavetta lanceolata*, family Rubiaceae,[31] as well as from the stem bark and leaves of the plant *Soulamea fraxinifolia*, family Simarubaceae.[36] Later it was isolated from the Tasmanian bryozoan *Costaticella hastata*,[15] from the New Zealand bryozoan *Cribricellina cribraria*,[16] and from *Hannoa chlorantha* root bark.[37]

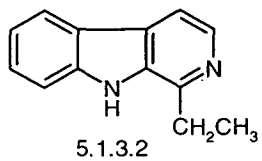


5.1.3.4

5.4.5.5 1-Ethyl-β-carboline (5.1.3.2).

The GCMS spectrum (Figure A5.4.5.5.1) showed m/z 197 (10), 196 (M^+, 72), 195 (100), 181 (5), 168 (23), 154 (8), 140 (10), 127 (5), 115 (7), matching with the literature data (m/z 196 (M^+, 77), 195 (100), 180 (8), 168 (29))[38] of 1-ethyl-β-carboline (5.1.3.2). So far 1-ethyl-β-carboline was isolated from the bark of *Aeschrion crenata* Vell. (*Picrasma crenata* (Vell.) Engl.),[39] from three different samples of *Hannoa klaineana* roots, family Simaroubacea,[38, 40] from the bark of the Indonesian plant *Picrasma javanica*.[41] This alkaloid has been previously isolated from marine organisms, first from the Tasmanian bryozoan

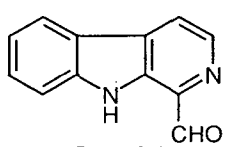
Costaticella hastata,[15] and later from the New Zealand bryozoan *Cribricellina cribraria*.[16] It was subsequently isolated from the wood of *Ailanthus malabarica*,[42] and from the root bark and root wood of *Brucea mollis* var. tonkinensis.[43]



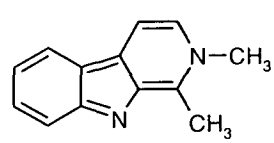
5.1.3.2

5.4.5.6 Compound no. 8.

The GCMS spectrum (Figure A5.4.5.6.1) of compound no. 8 showed m/z 197 (9), 196 (M^+, 49), 168 (52), 140 (26), 127 (11), 113 (17), 99 (14), 85 (61), 71 (64), 63 (13), 57 (100), 43 (85), 41 (53). A loss of 28 was accounted for by an formyl or ethyl group. The literature mass spectral data (m/z 197 (15), 196 (M^+, 64), 168 (100), 140 (54), 114 (24))[30] of 1-formyl-β-carboline was different from that of compound no. 8. The peak at 168 was the base peak in 1-formyl-β-carboline (5.4.5.6.1) but it was not the base peak in compound no. 8. Compound no. 8 is more likely to be an isomer of 1-formyl-β-carboline. The mass spectrum of neither 1-ethyl-β-carboline (5.1.3.2)[37] nor 1,2-dimethyl-β-carboline (5.4.5.6.2) (m/z 197 (19), 195 (37), 196 (100), 181 (20), 168 (10), 167 (7), 154 (32), 140 (3), 127 (10))[29] matched that of compound no. 8's spectrum. In view of the uncertainties of relying completely on mass spectral data, a further investigation that includes getting NMR data is warranted.



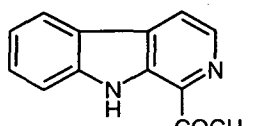
5.4.5.6.1



5.4.5.6.2

5.4.5.7 1-Acetyl-β-carboline (5.4.4.3.1).

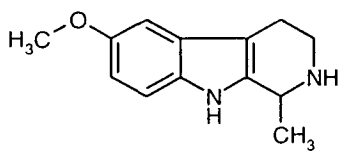
The GCMS spectrum (Figure A5.4.5.7.1) showed m/z 211 (14), 210 (M^+, 100), 182 (56), 168 (91), 167 (44), 140 (15), which matched the literature data[31] of 1-acetyl-β-carboline (5.4.4.3.1). This alkaloid was isolated from the bark and roots of *Ailanthus malabarica*, family Simarubaceae,[31] from the roots of *Picrasma quassiodes* Bennet (Simaroubacea),[44] from the stems of *Nauclea officinalis*,[45] and isolated as a metabolite of an actinomycete from the culture filtrate of *Streptomyces kasugaensis*,[46] from the sponge *Tedania ignis*,[47] as well as from the fern *Hypodematium squamuloso-pilosum*.[48]



5.4.4.3.1

5.4.5.8 Compound no. 9.

Compound no. 9 was isolated as 83.9% pure by GCMS. The GCMS spectrum (Figure A5.4.5.8.1) of compound no. 9 showed m/z 216 (M^+, 14), 197 (10), 196 (69), 195 (100), 181 (6), 169 (7), 168 (23), 167 (7), 154 (8), 140 (10), 127 (5), 115 (6). Compound no. 9's mass spectrum was different from the literature data[29] (m/z 216 (67), 201 (100), 187 (43), 172 (15), 158 (7), 144 (9), 130 (5), 115 (5), 100 (4)) of compound (5.4.4.6.1) whose molecular weight is 216. Compound no. 9 is unlikely to be 1,2,3,4-tetrahydro-6-methoxy-1-methyl-β-carboline (5.4.4.6.1) or an isomer of it. From the literature data so far the methoxy group was found to vary at position 5, 6 and 7 of the 1,2,3,4-tetrahydro-1-methyl-β-carboline derivative; all of the three known compounds were reported from several terrestrial plant species.[32]



5.4.4.6.1

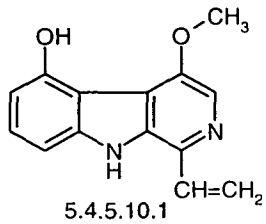
5.4.5.9 Compound no. 10.

The GCMS spectrum (Figure A5.4.5.9.1) of compound no. 10 showed m/z 218 (35), 217 (23), 216 (M^+, 100), 181 (22), 154 (14). Compound no. 10 is unlikely to be 1,2,3,4-tetrahydro-6-methoxy-1-methyl-β-carboline (5.4.4.6.1) or an isomer of it because compound no. 10's mass spectrum was substantially different from that of (5.4.4.6.1).[29]

5.4.5.10 Compound no. 11.

The GCMS spectrum (Figure A5.4.5.10.1) of compound no. 11 showed m/z 240 (M^+, 23), 227 (6), 226 (17), 225 (100), 224 (6), 207 (6), 193 (14), 181 (9), 167 (3), 154 (3), 140 (7), 113 (5) with losses of 15, 18, and 14. Compound no. 11 is not 5-hydroxydehydrocrenatine (5.4.5.10.1) but it might be an isomer of 5-hydroxydehydrocrenatine since the literature data of 5-hydroxydehydrocrenatin (m/z 240 (M^+, 100), 239 (36), 225 (12), 197 (23), 169 (14))[49] was only a little bit different from those of compound no. 11. The alkaloid

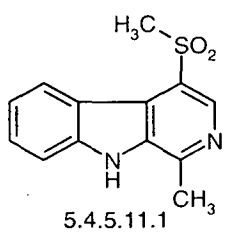
5-hydroxyhydrocrenatin itself came from the terrestrial plant *Picrasma javanica*, family Simarubacea.[49]



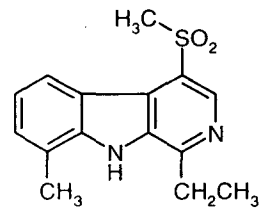
5.4.5.10.1

5.4.5.11 Compound no. 12.

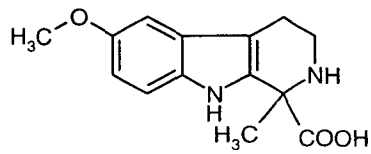
The GCMS spectrum (Figure A5.4.5.11.1) of compound no. 12 showed m/z 260 (M^+, 59), 181 (100), 154 (40), 127 918) with a loss of 79 for SO_2CH_3 group. Therefore, 1-methyl-4-methylsulfone-β-carboline or an isomer was proposed for compound no. 12. However, the structure of 1-methyl-4-methylsulfone-β-carboline (5.4.5.11.1) itself is closely related to the known compound, 1-ethyl-4-methylsulfone-β-carboline (5.1.3.6) found in the New Zealand bryozoan *Cribricellina cribraria*.[16]



5.4.5.11.1



5.1.3.6



5.4.4.6.2

5.4.5.12 Compound no. 13.

The GCMS spectrum (Figure A5.4.5.12.1) of compound no. 21 showed m/z 260 (M^+, 20), 219 (7), 218 (38), 217 (20), 216 (100), 181 (43), 179 (30), 154 (23), 127 (11). Compound no. 13 might be an isomer of 1,2,3,4-tetrahydro-1-carboxy-6-methoxy-1-methyl-β-carboline (5.4.4.6.2) because of losses of 44, that might be accounted for by a carboxy group, however, it was not compound (5.4.4.6.2).

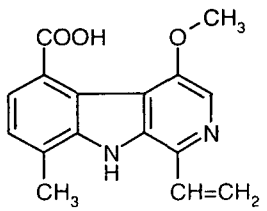
5.4.5.13 Compound no. 14.

The GCMS spectrum (Figure A5.4.5.13.1) of compound no. 14 showed m/z 274 (M^+, 12), 230 (31), 229 (28), 210 (13), 196 (38), 195 (100), 194 (7), 193 (18), 180 (5), 167 (14), 140 (11), 115 (9) with losses of 44 and 35. The literature data of compound (5.1.3.6) was reported from Prinsep *et al.* (m/z 274.0777 (M^+, 100), 246.0470 (12), 195.0931 (13), 168.0734)[16] which was different from compound no. 14's mass spectrum. Compound no. 14

is unlikely to be 1-ethyl-4-methylsulfone-β-carboline (5.1.3.6)[16] but it might be an isomer of it.

5.4.5.14 Compound no. 15.

The GCMS spectrum (Figure A5.4.5.14.1) of compound no. 15 showed m/z 286 (8), 285 (23), 284 (M^+, 100), 283 (35), 269 (70), 239 (11), 238 (11), 197 (3), 196 (8), 195 (50), 194 (59), 193 (37), 192 (3), 182 (11), 168 (16), 73 (31). Losses of 15, 31 and 44 were accounted for by methyl, methoxy and carboxy groups being attached to 1-vinyl-β-carboline (5.1.3.4), molecular weight of 194. Therefore, an isomer of 5-carboxy-4-methoxy-8-methyl-1-vinyl-β-carboline (5.4.5.14.1) was proposed for compound no. 15.



5.4.5.14.1

5.4.5.15 Compound no. 16.

The GCMS spectrum (Figure A5.4.5.15.1) of compound no. 16 showed m/z 328 (M^+, 7), 315 (7), 314 (20), 313 (67), 232 (19), 231 (370, 230 (71), 229 (100), 202 (19), 179 95), 166 (8), 145 (12), 132 (19), 129 (23), 117 (38), 73 (41). No match could be found between this data and that found in databases or in the literature.

5.4.5.16 Other compounds in the pycnogonid detected by LCMS.

Two fractions from the pycnogonid *Pseudopallene ambigua* were subjected to a preliminary analysis by using ESI LCMS on a C18 column eluted under isocratic conditions with methanol-water (80:20) with 0.1% ammonium acetate for 4 mins and gradient to methanol-water (95:5). Some compounds that were not detected by GCMS were found by using LCMS. These compounds, which were detected by LCMS (Figure A5.4.5.16.1, A5.4.5.16.2) but not by GCMS, had molecular weights of 184, 198, 212, 228, 266, 282, 350, 364, and 378. It would be interesting to see their complete structures from this preliminary investigation.

5.5 Conclusion.

Two known metabolites, 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (5.1.1.3), and 1,8-dihydroxyanthraquinone (5.1.1.5) were isolated from the bryozoan *Watersipora subtorquata*.

Tambjamines A (5.2.1), B (5.2.2), C (5.1.2.6), D (5.2.3), E (5.1.2.7), G (5.1.2.2), H (5.1.2.3), and J (5.1.2.5), as well as the blue pigment (molecular weight of 336), and some unidentified compounds (molecular weight of 259, 361, and 439) were found in both the bryozoan *Bugula dentata* and its associated nudibranch *Tambja verconis*.

Several derivatives of β-carbolines were detected from the bryozoan *Cribricellina rufa* and its associated pycnogonid *Pseudopallene ambigua*. It is interesting that both organisms contained the same as well as different derivatives of β-carbolines. Norharman (5.4.5.1.1), pavettine (5.1.3.4), 1-ethyl-β-carboline (5.1.3.2) and 1-acetyl-β-carboline (5.4.4.3.1), together with the other ten unidentified structures (the isomer of harman (compound no. 7, MW 182), isomer of 1-formyl-β-carboline (compound no. 8, MW 196), and compound nos. 9-16) were found only in the pycnogonid. Similarly, compound no. 1 (MW 222), compound no. 4 (MW 254), compound no. 5 (MW 258), and compound no. 6 (MW 288) were found in the bryozoan only. Harman (5.1.3.1), the isomer of dehydrocrenatine (compound no. 2, MW 224), and compound no. 3 (MW 238) were present in both organisms. This preliminary study points out that a further investigation of this interesting aspect of marine chemical ecology should be carried out and so has been recorded in detail here. Questions to be answered include: what is the variability of the secondary metabolites in the bryozoan and the pycnogonid—does it change between individuals, with time or with location? Does the pycnogonid sequester these compounds and if so why? Is the bryozoan *Cribricellina rufa* the sole source of food for the pycnogonid and if so how and why does the pycnogonid apparently transform some of the bryozoan's β-carboline alkaloids? If the pycnogonid consumes other species of bryozoans, which are they and what is their chemistry? It seems that no chemical work has been investigated on the bryozoan species *Cribricellina rufa* and the pycnogonid *Pseudopallene ambigua* before. However, the bryozoan *Cribricellina cribraria* from New Zealand sample has been examined as mentioned earlier.[16]

5.6 Experimental.

5.6.1 Collection.

The bryozoan *Watersipora subtorquata* was collected from Triabunna on 30th July 1998 and 21st July 1999 by scuba diving. The bryozoan *Bugula dentata* and the nudibranch *Tambja verconis*, together with other nudibranchs, *Ceratosoma brevicaudatum*, *Chromodoris tasmaniensis* and *Chromodoris epicuria* were collected at the same place and time from Triabunna on 21st July 1999. The bryozoan *Cribricellina rufa* and the pycnogonid *Pseudopallene ambigua* were collected at Bichino, old jetty area on 27th Oct. 1998 and 6th Sept. 1999 by scuba diving.

5.6.2 Extraction procedure.

Freeze-dried *Watersipora subtorquata* 155.4 g was extracted with methanol-dichloromethane (3:1). Ethyl acetate was used to dissolve the methanol-dichloromethane crude extract 18.141 g, 11.67% yield (base on the bryozoan dry weight).

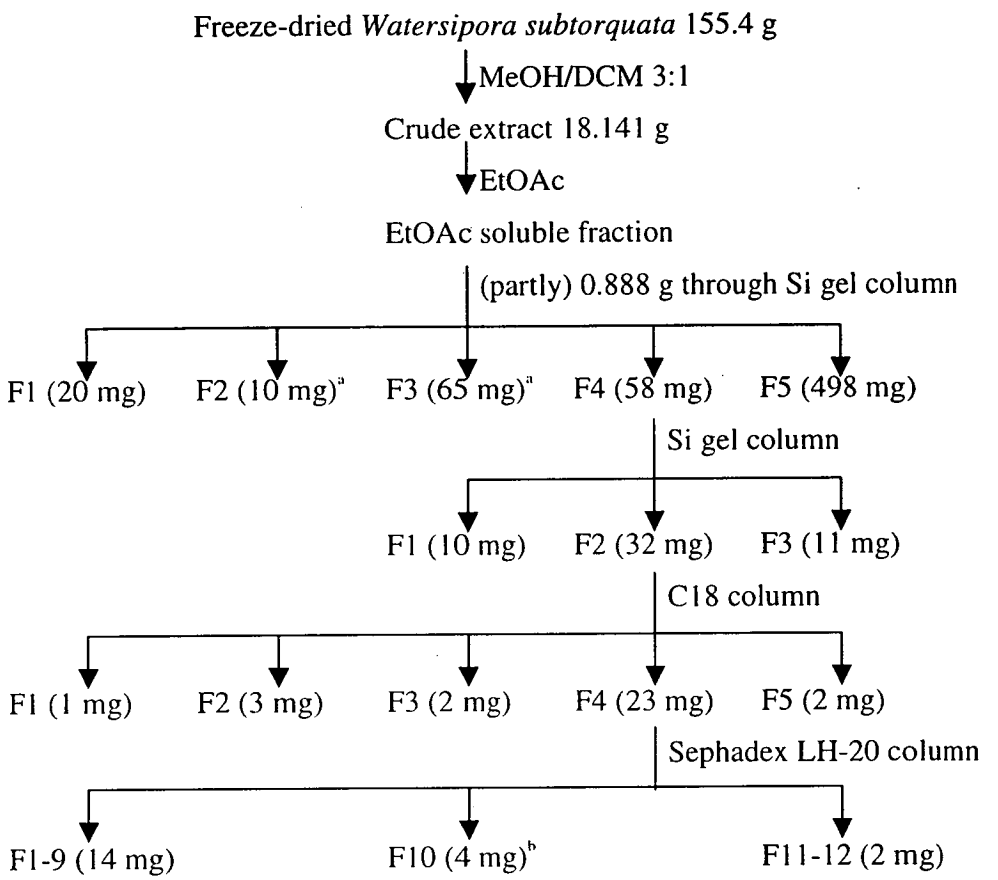
The wet bryozoan *Bugula dentata* 39.782 g was extracted straight away with methanol-dichloromethane (1:1) to give a viscous crude extract 2.226 g, 5.60% yield (base on the bryozoan wet weight).

Freeze-dried *Cribricellina rufa* 32.3 g was extracted with methanol-dichloromethane (1:1) to give a viscous crude extract 0.223 g, 0.69% yield (base on the bryozoan dry weight), showing a positive test to Meyer's reagent.

Freeze-dried nudibranch *Tambja verconis* 19.477 g was extracted with dichloromethane to give a viscous crude extract 1.697 g, 8.71% yield (base on the nudibranch dry weight).

The wet pycnogonid *Pseudopallene ambigua* 3.431 g was extracted straight away with methanol-dichloromethane (1:1) to give yellow oil crude extract 0.286 g, 8.34% yield (base on the pycnogonid wet weight).

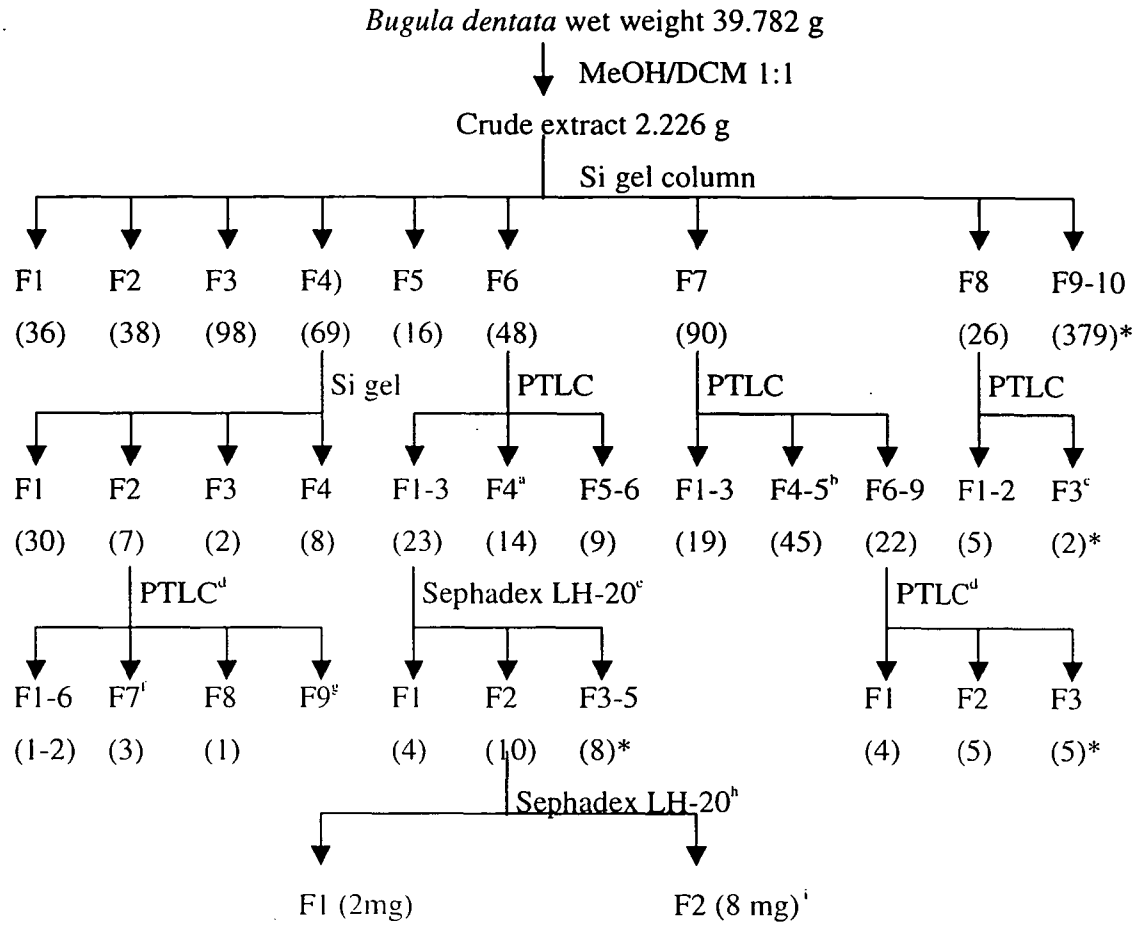
5.6.3 Separation procedure.



[a]contained the known compound (5.1.1.5).

[b]the known compound (5.1.1.3).

Scheme 5.6.3.1 Separation procedure of the bryozoan *Watersipora subtorquata*.



* milligram unit.

[a] tambjamine G.

[b] tambjamines C, D, G, H, J, and the MW 259 compound.

[c] MW 336.

[d] 10% MeOH in EtOAc-DCM (1:4).

[e] MeOH

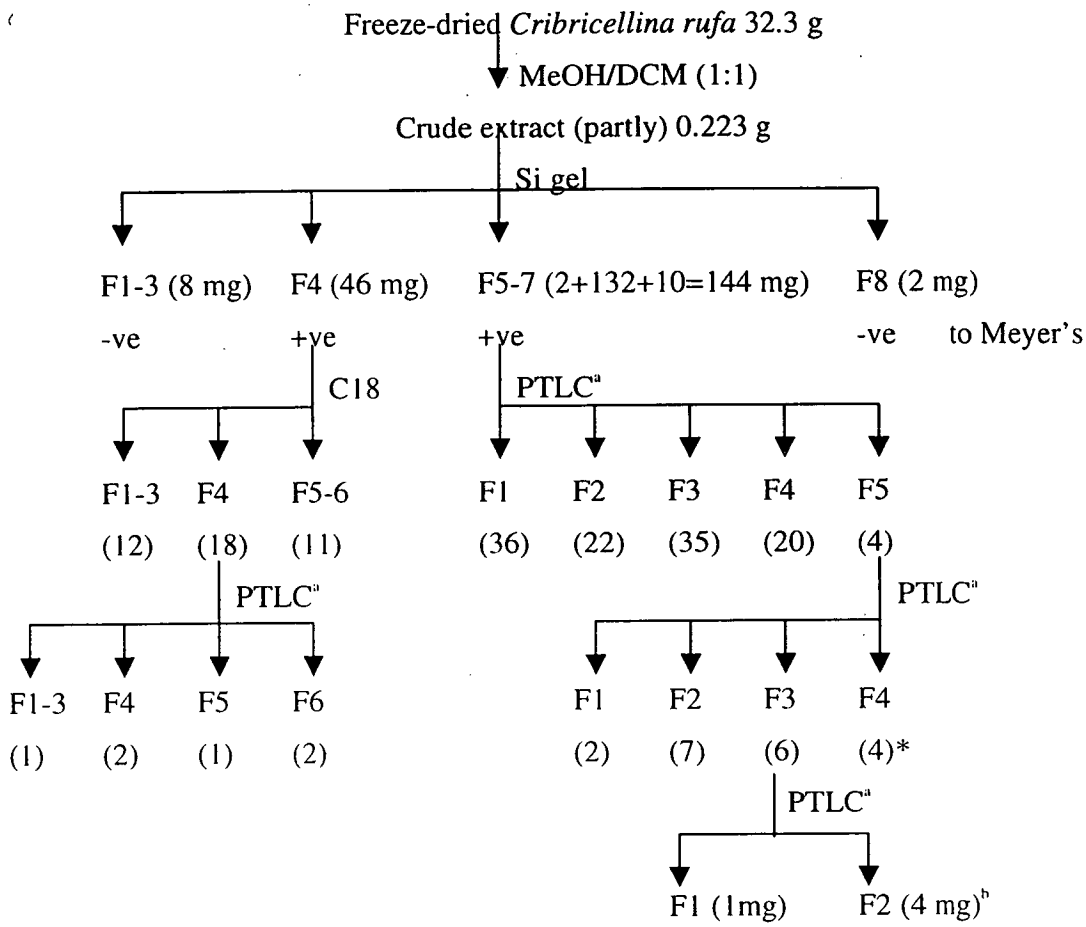
[f] Tambjamines A and B.

[g] Tambjamine A.

[h] 50% MeOH-DCM.

[i] blue pigment MW 336.

Scheme 5.6.3.2 Separation procedure of the bryozoan *Bugula dentata*.

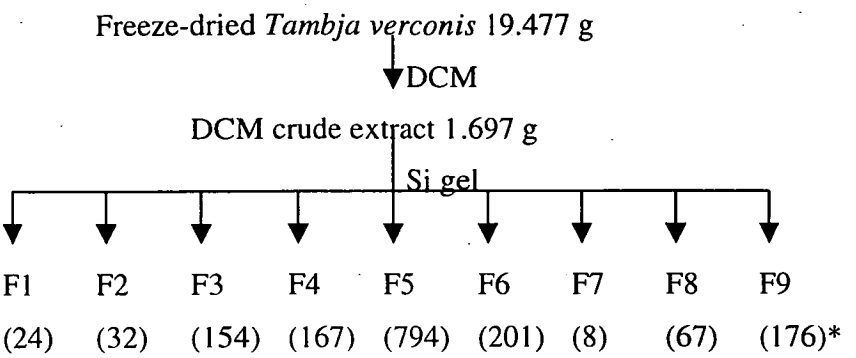


* milligram unit.

[a] 10% MeOH in EtOAc-DCM (1:4).

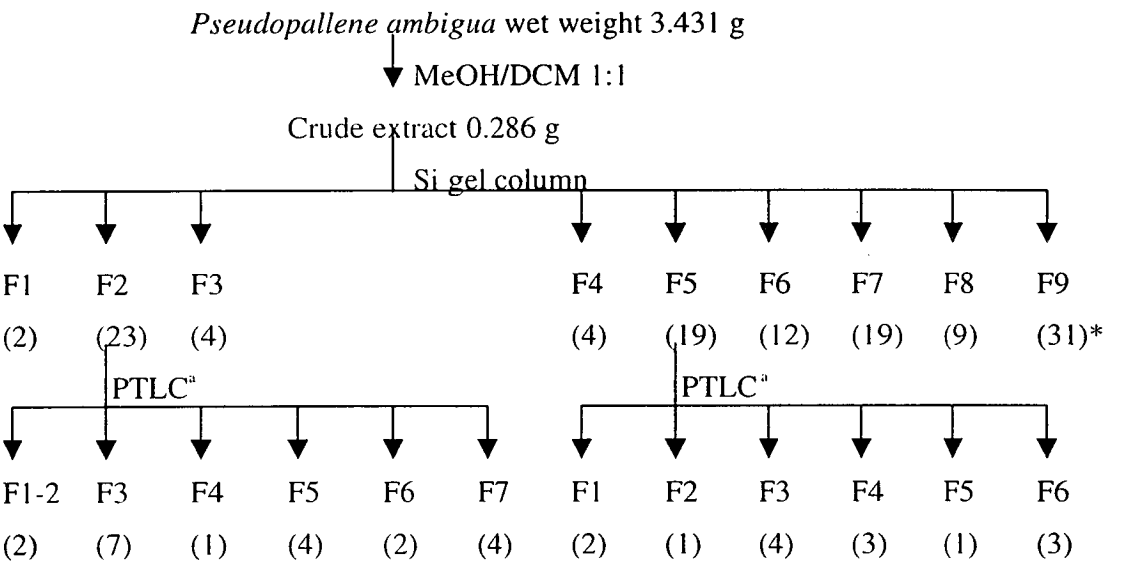
[b] Harman.

Scheme 5.6.3.3 Separation procedure of the bryozoan *Cribricellina rufa*.



* milligram unit.

Scheme 5.6.3.4 Separation procedure of the nudibranch *Tambja verconis*.



* milligram unit.

[a]5% MeOH in EtOAc-DCM (1:4).

Scheme 5.6.3.5 Separation procedure of the pycnogonid *Pseudopallene ambigua*.

5.6.4 Characterization of the compounds as follows.

5,7-Dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9*bc*]thiophene (5.1.1.3) was isolated as red colour pigment and its ^{1}H NMR data was consistent with the literature.[9]

1,8-Dihydroxyanthraquinone (5.1.1.5) was isolated as yellow oil and its ^{1}H NMR data was consistent with the literature.[11]

Tambjamines A (5.2.1), B (5.2.2), C (5.1.2.6), D (5.2.3), E (5.1.2.7), G (5.1.2.2), H (5.1.2.3), and J (5.1.2.5) were found and their molecular weights were consistent with the literature.[13, 23]

Harman (5.1.3.1) was isolated (6 mg) and the GCMS as well as the ^{1}H NMR data were consistent with the literature.[28, 29, 30]

Norharman (5.4.5.1.1), pavettine (5.1.3.4), 1-ethyl-β-carboline (5.1.3.2), and 1-acetyl-β-carboline (5.4.4.3.1) were found and their GCMS data were consistent with the literature.[29, 30, 33, 34]

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Chapter 6. Brown Algae.

6.1 General introduction and secondary metabolites from brown algae.

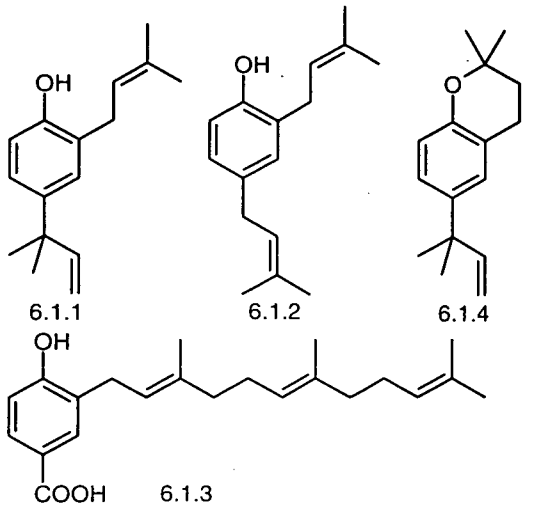
The brown algae *Bellotia eriphorum*, *Sporochnus comosus*, *Sporochnus* species and *Perithalia caudata* have been investigated in this study. All of these brown algae belong to the phylum Phaeophyta, class Phaeophyceae, order Sporochnales, and family Sporochnaceae.[1]

In 1981 a report about phenolic substances from marine organisms was published, 42 of a total 126 phenolic substances were from brown algae in the order Fucales, Dictyotales, Laminariales, and Sphacelariales. These were phloroglucinol oligomers and polyprenyl hydroquinones. This review classified all types of phenols in nature as shikimic acid-derived simple phenols, oligophenols, acetate-derived phenols, mevalonate-derived phenols, and polyprenyl phenols.[2]

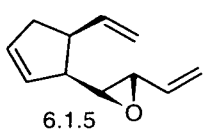
This review will discuss typical secondary metabolites from the brown algae in the genera *Sporochnus* and *Perithalia* using SciFinder Scholar covering structure determination, method of isolation, synthesis, and biological activity where possible from the period of 1979 to 2000.

Blackman *et al.* first isolated a new phenol, 4-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)phenol (6.1.1) from the brown alga *Perithalia caudata*, which was collected at Nine Pin Point, D'Entrecasteaux Channel, Tasmania, Australia.[3] A second component (9% from the first report) was then later identified and obtained pure in 1988 as 2,4-bis(3-methylbut-2-enyl)phenol (6.1.2).[4]

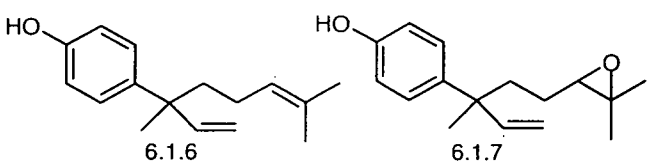
In 1994 a new sesquiterpene phenol, 4-hydroxy-3-(1'-((2'*E*, 6'*E*)-3',7',11'-trimethyl-2',6',10'-dodecatrienyl))-benzoic acid (6.1.3) and two known compounds, (6.1.1), (6.1.4) were isolated by Rochfort and Capon from *Perithalia caudata*, which was collected offshore from Flinders, Victoria, Australia.[5]

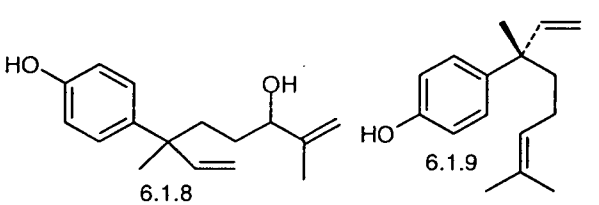


A new gamete-releasing and gamete-attracting pheromone, (+)-caudoxirene (6.1.5) was found from the brown alga *Perithalia caudata*[6] and its structure, including absolute configuration, were confirmed by synthesis.[7,8]



Prenylated phenols, sporochnol A (6.1.6), B (6.1.7), and C (6.1.8) were isolated from the Caribbean brown alga *Sporochnus bolleanus*.[9] The absolute configuration of (+)-Sporochnol A (6.1.9), a fish deterrent, was shown to be *S* by a stereocontrolled synthesis of its enantiomer from (*S*)-epichlorohydrin.[10]





Up till now, an investigation of the brown alga genus *Bellotia* has not been reported. Here, a study of secondary metabolites from the brown alga, *Bellotia eriphorum*, *Sporochnus comosus*, *Sporochnus stylosus*, *Sporochnus moorei*, *Sporochnus* species and

Perithalia caudata were compared. All the species of the brown alga discussed in this chapter, especially *Sporochnus* species identified by comparing the appearance to the reference.[1] The identification was tentative.

6.2 Results and discussion.

Bellotia eriophorum was collected from Gordon, Tasmania on 27th Mar. 1997 by scuba diving. The freeze-dried alga was extracted with dichloromethane and methanol. The dichloromethane extract was purified by an open-column flash Si gel chromatography eluted with petroleum ether and increasing percentage of ethyl acetate to give six fractions. The second and third fractions from ethyl acetate-petroleum ether (20-40%) were further purified by MPLC to afford a mixture of phenols (6.1.1) 5.1% and (6.1.2) 93.0%. Purity was based on the total ion chromatogram obtained by GCMS.

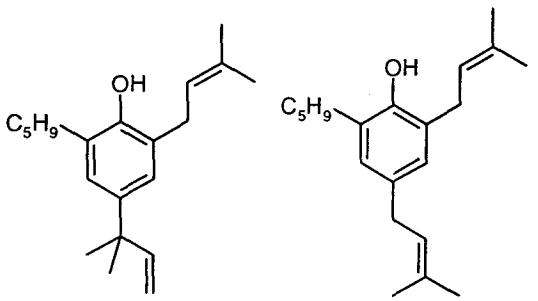
Sporochnus species was collected from Gordon on 27th Mar 1997 by scuba diving. The freeze-dried sample was extracted with dichloromethane and methanol. The dichloromethane extract contained only phenol (6.1.2). The total ion chromatogram was not good enough to report its percentage.

Sporochnus comosus was collected from Spikey Bridge on 19th Nov 1997 by scuba diving. The freeze-dried sample was extracted with dichloromethane and methanol. The dichloromethane extract contained phenols (6.1.1) 88.3%, (6.1.2) 1.1% and an unidentified compound MW 248 (2.0% total ion area), which corresponds to 18 more than that of (6.1.1) or (6.1.2) with MW 230. The mass of 18 could be accounted for by water, being added to the olefinic side chain of the compound (6.1.1) or compound (6.1.2).

Sporochnus stylosus was collected from Spikey Bridge on 16th Feb 1998 by scuba diving. The freeze-dried sample was extracted with dichloromethane and methanol. The dichloromethane extract contained phenols (6.1.1) 88.0% and the compound MW 248.

Sporochnus moorei was collected from Spikey Bridge on 16th Feb 1998 by scuba diving. The freeze-dried sample was extracted with dichloromethane and methanol. The dichloromethane extract contained phenol (6.1.1) 5.6%, the compound MW 248, 14.0%, as well as a compound MW 298 isomer no. 1, 59.9% and a compound M^+ 298 isomer no. 2, 1.7% total ion area. The phenols MW 298 have 68 more mass units that (6.1.1) or (6.1.2). The extra mass could be explained by these compounds having an additional pentenyl group ($-C_5H_9$) of some type, most likely identical to one of those already present in (6.2.1).

The extra group is most probably attached *ortho* to the phenolic group in either (6.1.1) or (6.1.2). Thus the phenol isomers nos. 1-2 are most likely to be compound (6.2.1) or one of its isomers, otherwise compound (6.2.2) or one of its isomers.



6.2.1 6.2.2

Perithalia caudata was collected from Gordon on 18th Feb 1997 by scuba diving. Freeze-dried sample was extracted with dichloromethane and methanol. The dichloromethane extract contained phenols (6.1.1) 85.4% and (6.1.2) 1.4% total ion area.

6.3 Conclusion.

The brown algae *Bellotia*, *Perithalia* and *Sporochnus* belong to the same order Sporochnales and for the first time, they have all been found to contain closely related secondary metabolites. Two known phenols (6.1.1-6.1.2) were found in the brown algae *Bellotia eriphorum*, *Perithalia caudata* and *Sporochnus* species. Secondary metabolites from the brown alga *Bellotia eriphorum* have not been reported previously in the literature, unfortunately, only known metabolites were found in this study. The four *Sporochnus* algae examined were chemically different and may possibly belong to different species. Three new phenols were noted for the brown algae *Sporochnus* species. This preliminary work has not been completed because of the difficulty of obtaining more of the algae due to their seasonal growth pattern and scarcity. Nevertheless these results indicate that this genus should be examined in more detail.

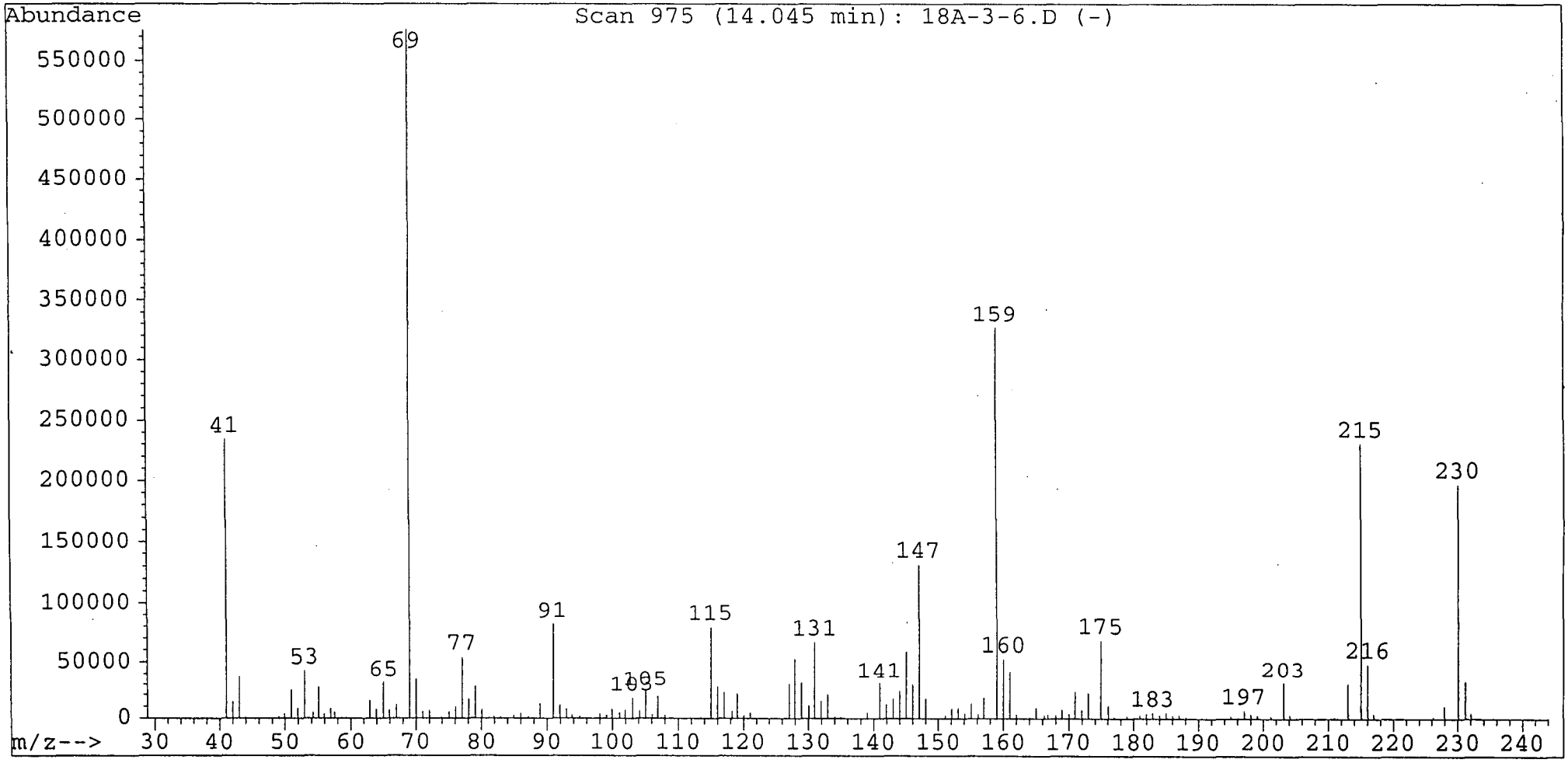


Figure 6.2.1 GCMS spectrum of 4-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)phenol (6.1.1).

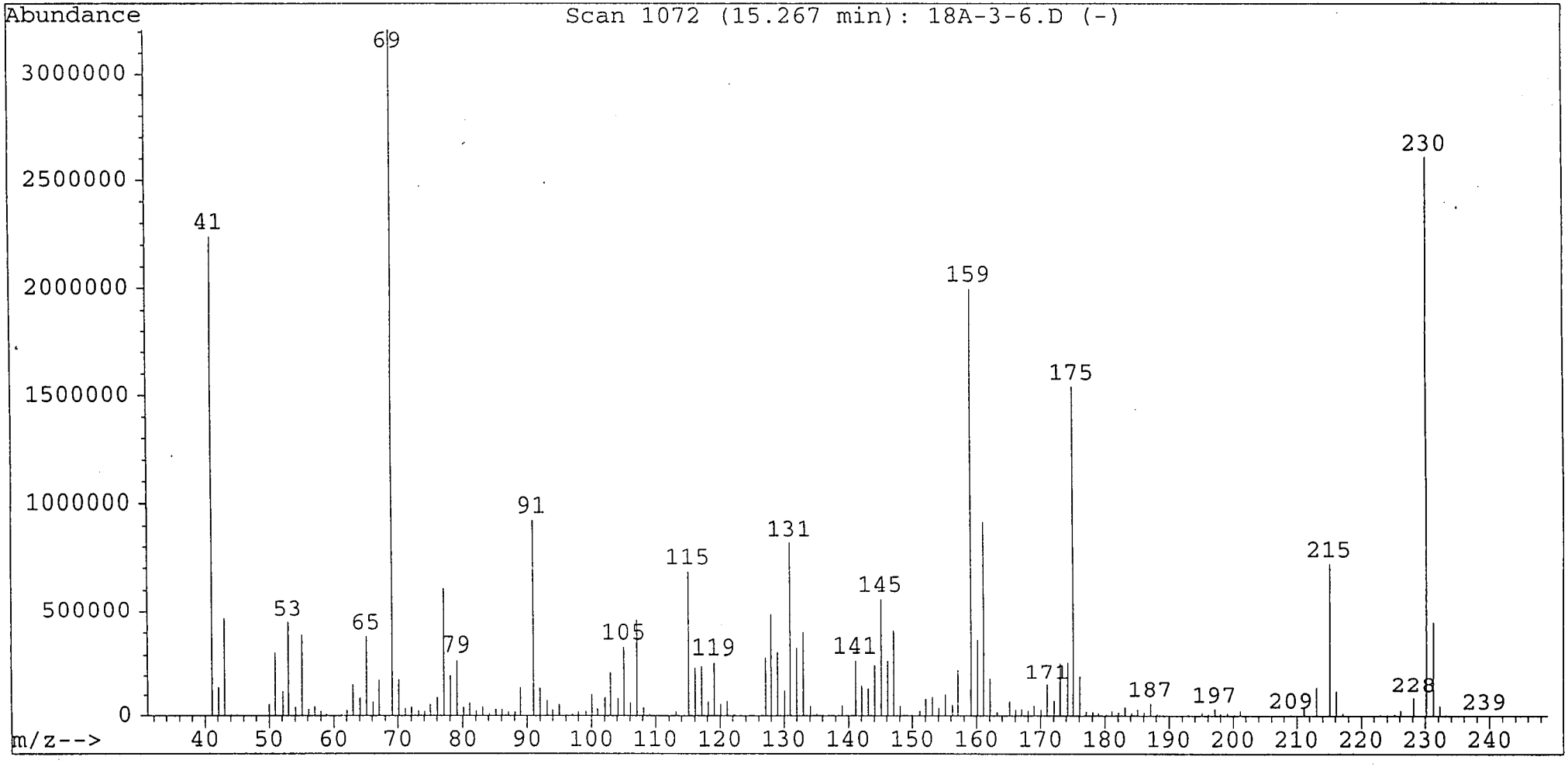


Figure 6.2.2 GCMS spectrum of 2,4-bis(3-methylbut-2-enyl)phenol (6.1.2).

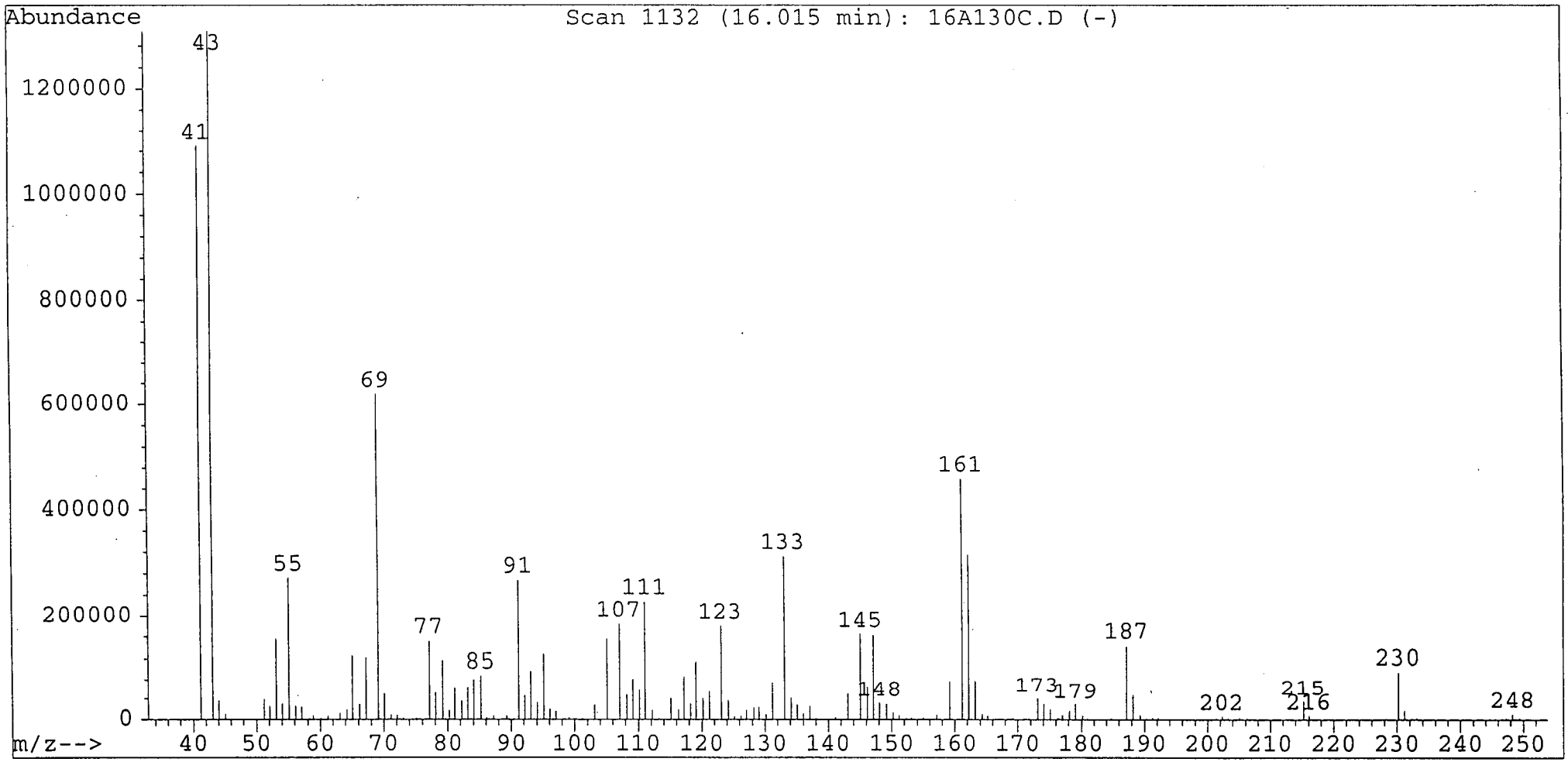


Figure 6.2.3 GCMS spectrum of the unidentified phenol MW 248.

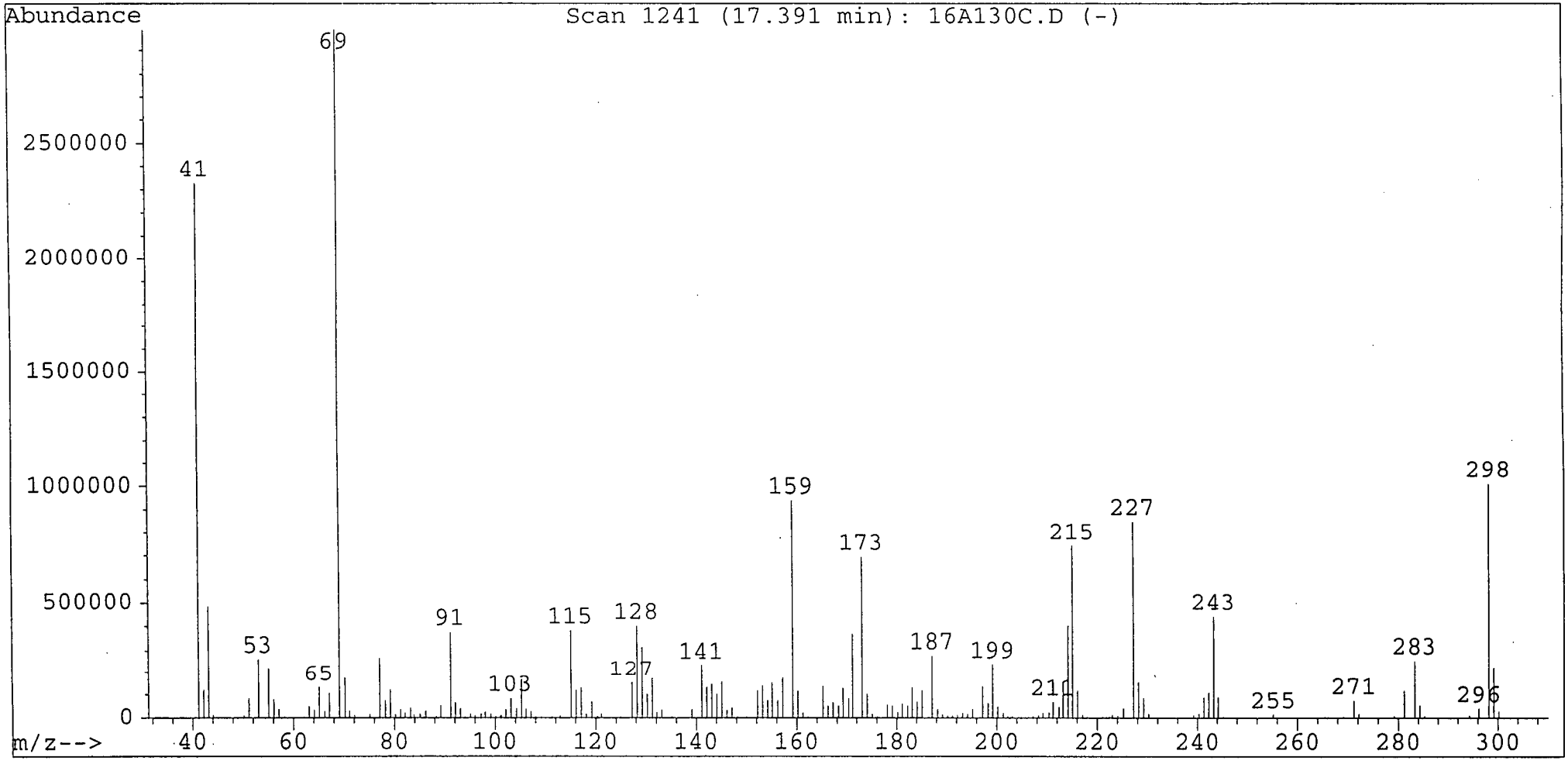


Figure 6.2.4 GCMS spectrum of the unidentified compound MW 298 isomer no. 1.

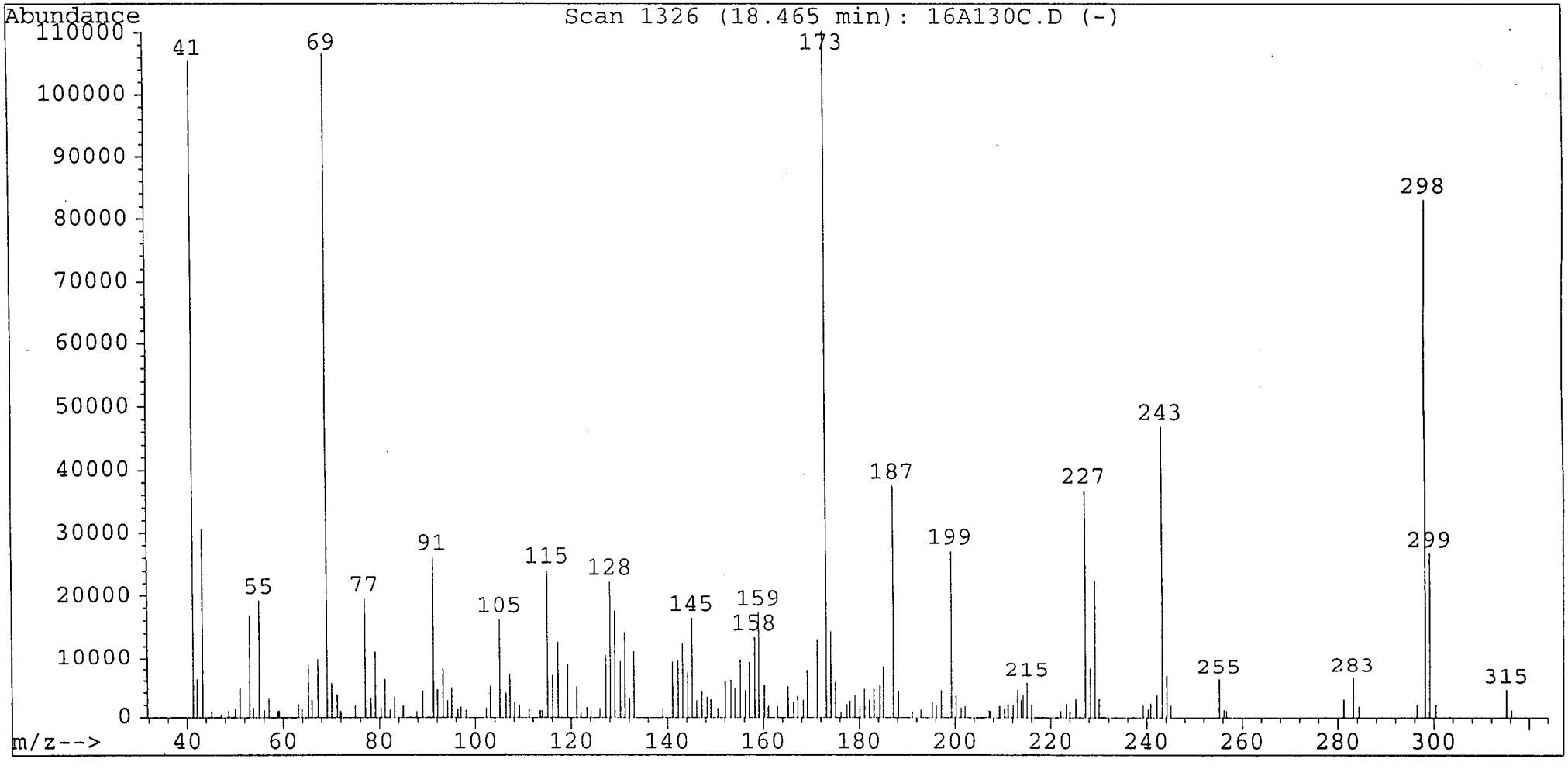


Figure 6.2.5 GCMS spectrum of the unidentified compound MW 298 isomer no. 2.

6.4 Experimental.

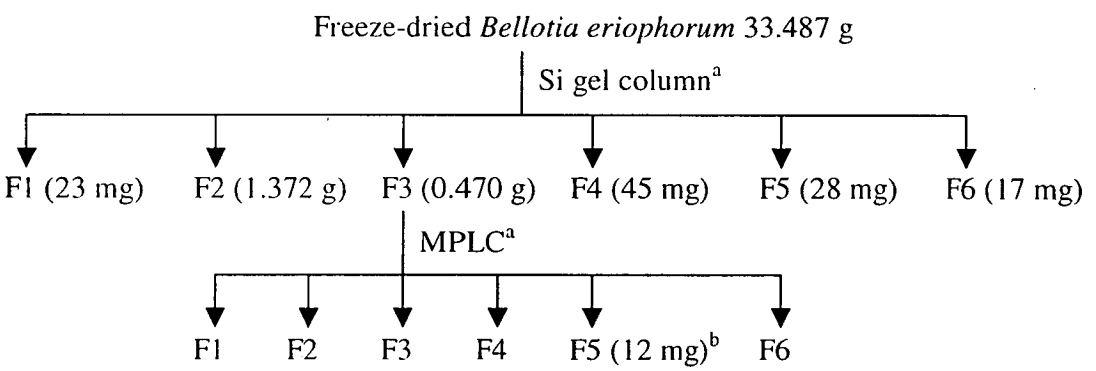
6.4.1 Collection.

Bellotia eriphorum was collected at Gordon, Tasmania on 27th Mar 1997. *Perithalia caudata* was collected at Gordon on 27th Mar 1997. *Sporochnus* species was collected at Gordon on 27th Mar 1997. *Sporochnus comosus* was collected at Spikey Bridge on 19th Nov 1997. In the collection on 16th Feb 1998 at Spikey Bridge, the *Sporochnus* were sorted into two types because of their appearance. These were tentatively identified by Christian Narkowicz as being different species, *Sporochnus stylosus* and *Sporochnus moorei*. The voucher specimens were made and are stored in the School of Chemistry's collection.

6.4.2 Extraction procedure.

The brown algae were sorted, frozen after collection and then freeze-dried. The dried materials were separately extracted with dichloromethane and later with methanol. Both dichlormethane and methanol extracts were concentrated using a rotary evaporator at a temperature below 30 ^{0}C to give dark brown tars.

6.4.3 Separation procedure.



[a] Petroleum ether and increasing polarity with dichloromethane and ethyl acetate percentage.

[b] Contained phenols 6.1.1 and 6.1.2.

Scheme 6.4.3.1 Separation procedure of the brown alga *Bellotia eriphorum*.

Note: In the case of *Sporochnus* species and *Perithalia caudata*, only preliminary study was performed by GCMS.

6.4.4 Characterization of 4-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)phenol (6.1.1) and 2,4-bis(3-methylbut-2-enyl)phenol (6.1.2).

4-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)phenol (6.1.1) and 2,4-bis(3-methylbut-2-enyl)phenol (6.1.2) were isolated as a mixture of 6.1.1 (5.1%) and 6.1.2 (93.0%) by total ion area from the brown alga *Bellotia eriphorum*. The GCMS spectra of phenols (6.1.1-6.1.2) were consistent with the literature.[4] The compound MW 248 showed m/z 248 (M^+, 1), 230 (9), 215 (3), 187 (13), 161 (38), 133 (25), 111 (20), 91 (78), 69 (49), 55 (22), 43 (100), 41 (83). The compound MW 298 isomer no. 1 showed m/z 298 (M^+, 34), 283 (9), 243 (15), 227 (29), 215 (25), 173 (23), 159 (32), 128 (14), 115 (13), 91 (13), 77 (9), 69 (100), 41 (78) and the compound MW 298 isomer no. 2 showed m/z 298 (M^+, 74), 283 (6), 243 (43), 227 (33), 215 (5), 199 (25), 187 (35), 173 (100), 159 (16), 145 (14), 128 (21), 115 (23), 105 (13), 91 (22), 77 (16), 69 (97), 41 (97).

6.5 References.

[1] Womersley, H. B. S. *The Marine Benthic Flora of Southern Australia, Part II*, South Australian Government Printing Division, Adelaide, **1987**, pp. 273-289.

[2] Higa, T. (Scheuer, P. J. ed.) *Marine Natural Products, Chemical and Biological Perspectives*, vol. IV, Academic Press, 1981, pp. 93-145.

[3] Blackman, A. J.; Dragar, C.; Wells, R. J. *Aust. J. Chem.* **1979**, *32*, 2783-2786.

[4] Blackman, A. J.; Rogers, G. I.; Volkman, J. K. *Phytochemistry* **1988**, *27*, 3686-3687.

[5] Rochfort, S. J.; Capon, R. J. *J. Nat. Prod.* **1994**, *57*, 849-851.

[6] Muller, D. G.; Boland, W.; Becker, U.; Wahl, T. *Biol. Chem. Hoppe-Seyler* **1988**, *369*, 655-659.

[7] Wirth, D.; Boland, W. *Helv. Chim. Acta* **1990**, *73*, 916-921.

[8] Wirth, D.; Boland, W.; Muller, D. G. *Helv. Chim. Acta* **1992**, *75*, 751-758.

[9] Shen, Y. C.; Tsai, P. I.; Fenical, W.; Hay, M. E. *Phytochemistry* **1993**, *32*, 71-75.

[10] Takahashi, M.; Shioura, Y.; Murakami, T.; Ogasawara, K. *Tetrahedron: Asymmetry* **1997**, *8*, 1235-1242.

Appendix A

List of some mass spectra in chapter 5:

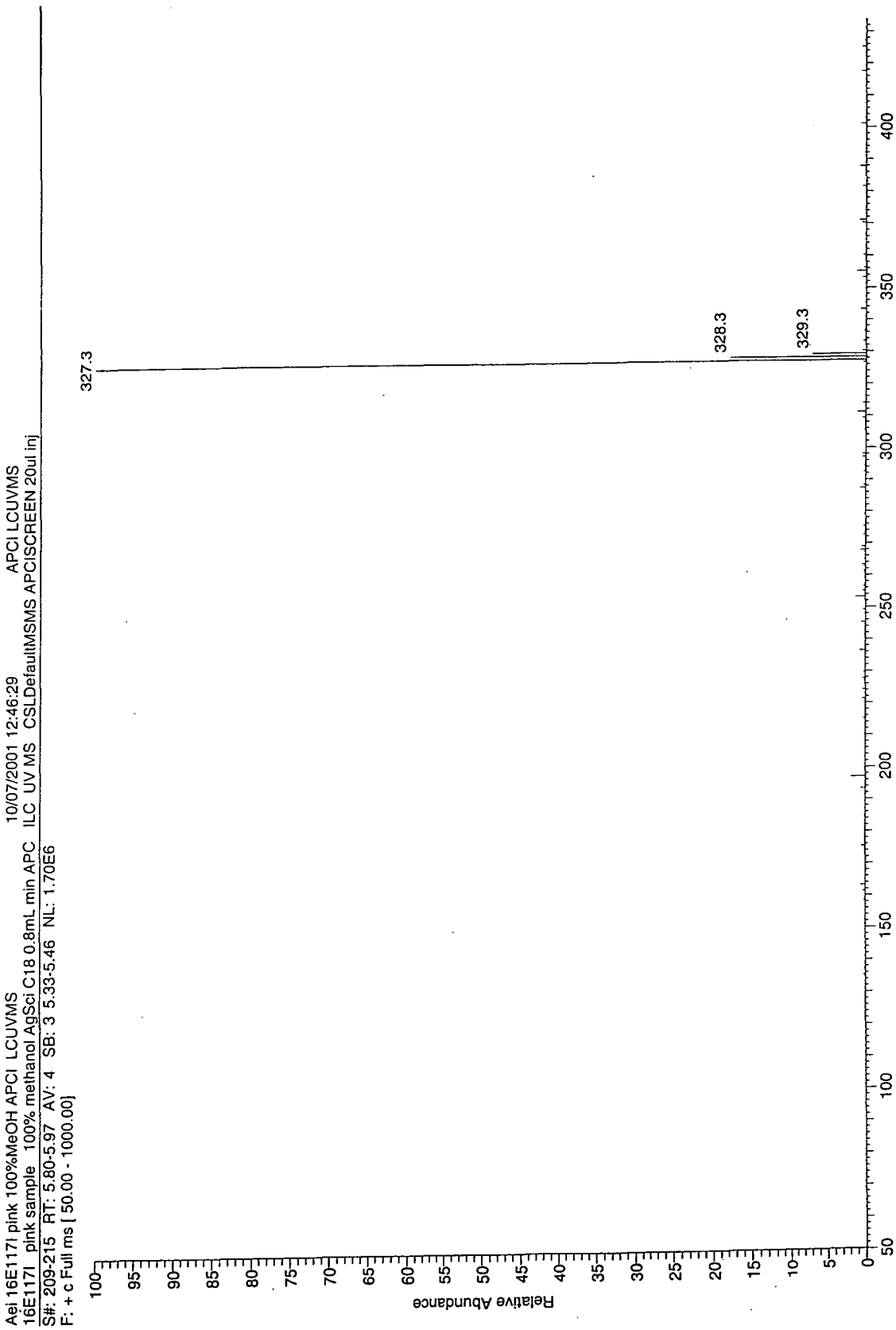


Figure A5.4.1.1.1 APCI LCMS spectrum of 5,7-dihydroxy-1-methoxycarbonyl-6 oxo-6*H*-anthra[1,9-*bc*]thiophene (5.1.1.3).

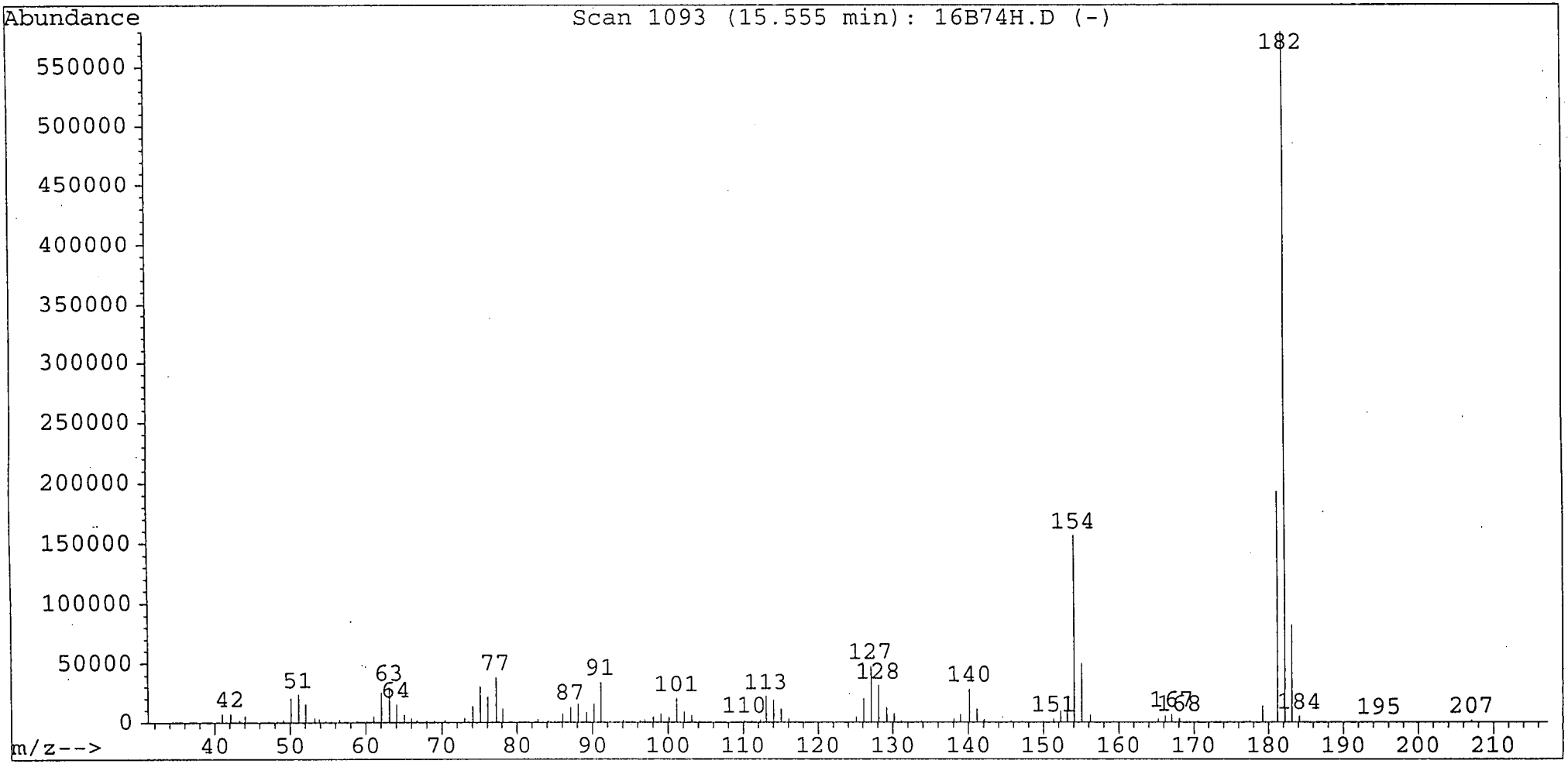


Figure A5.4.4.1.1 GCMS spectrum of harman (5.1.3.1).

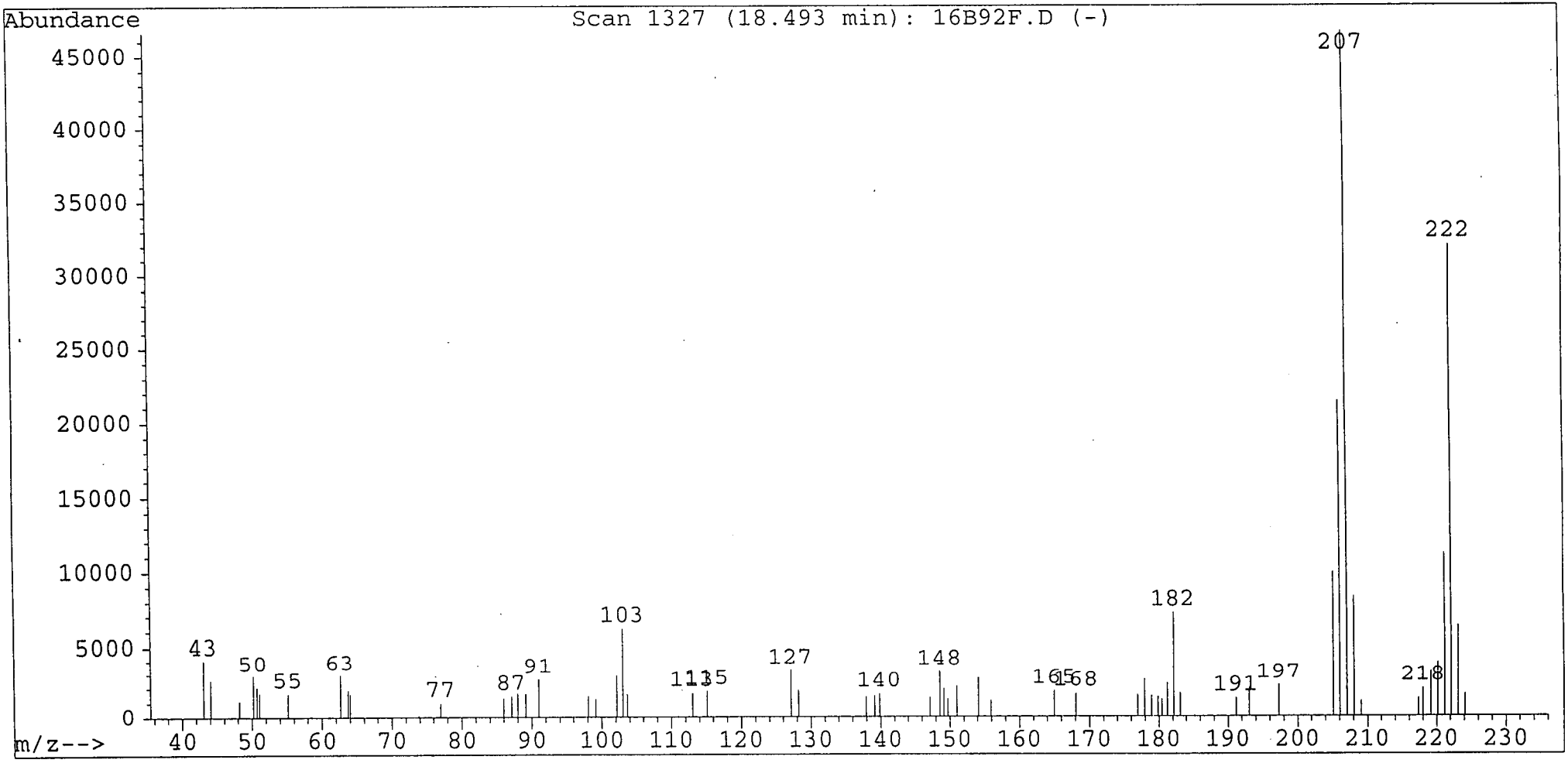


Figure A5.4.4.2.1 GCMS spectrum of compound no. 1 (MW 222).

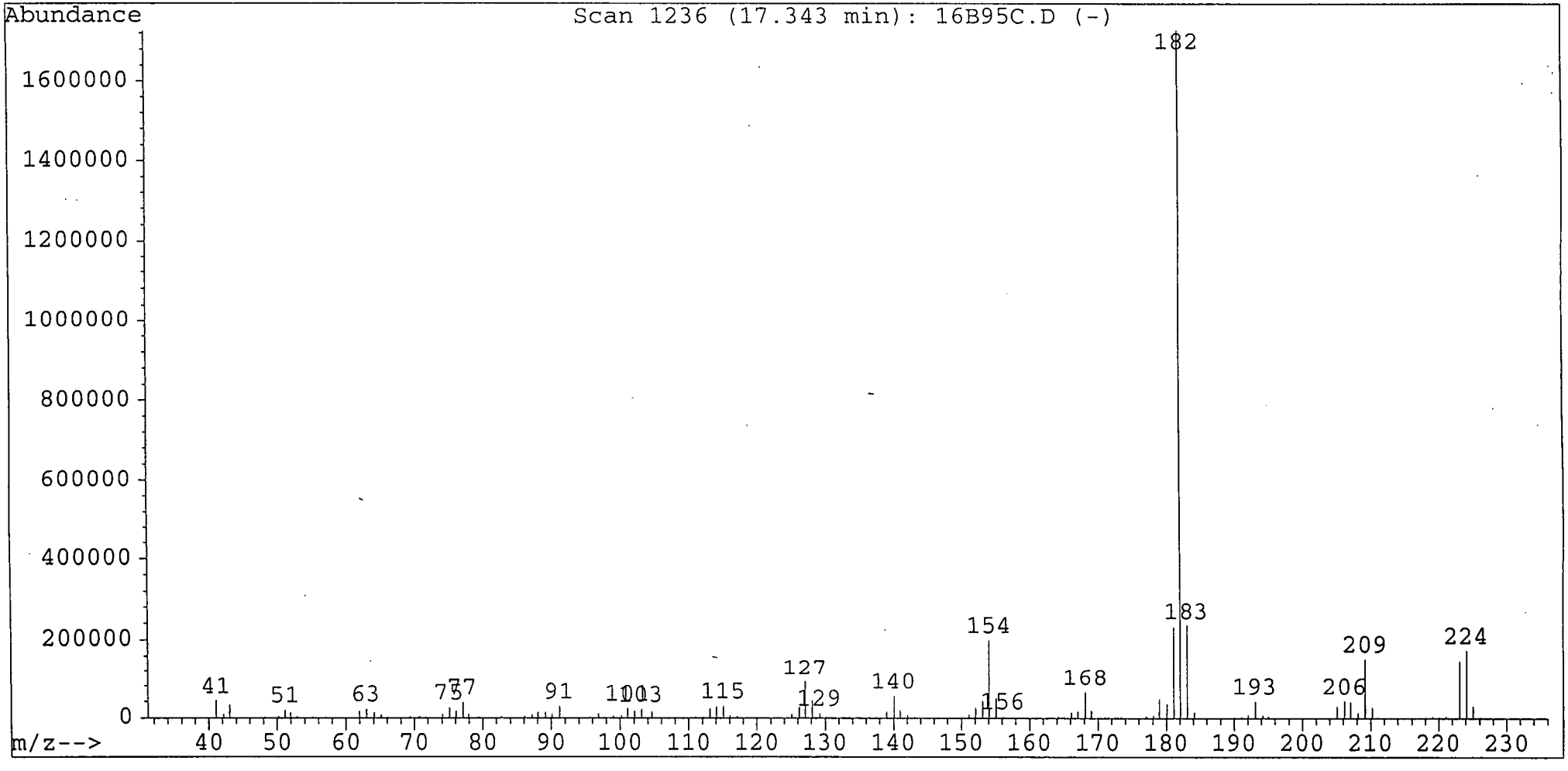


Figure A5.4.4.3.1 GCMS spectrum of compound no. 2 (MW 224).

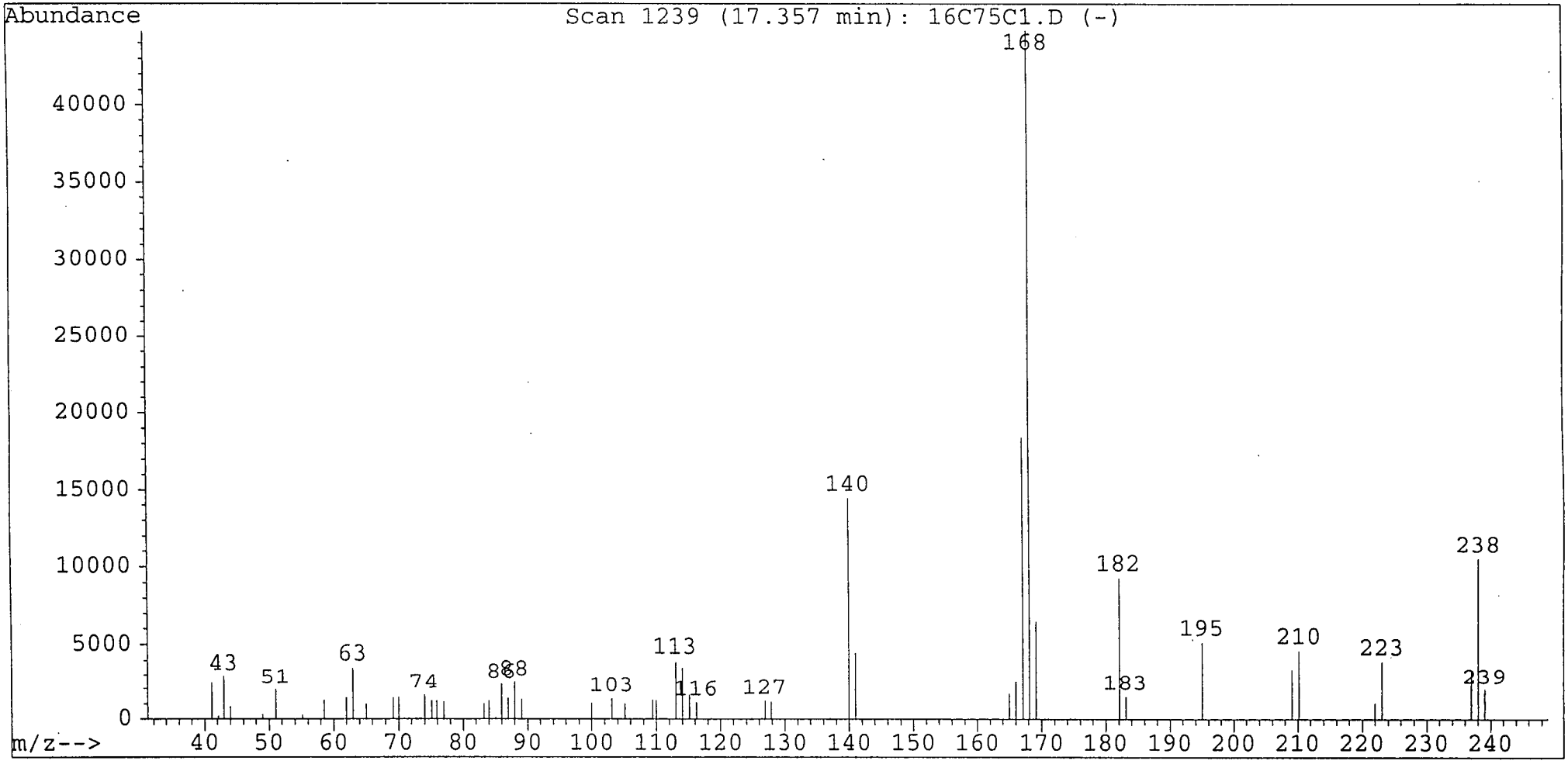


Figure A5.4.4.4.1 GCMS spectrum of compound no. 3 (MW 238).

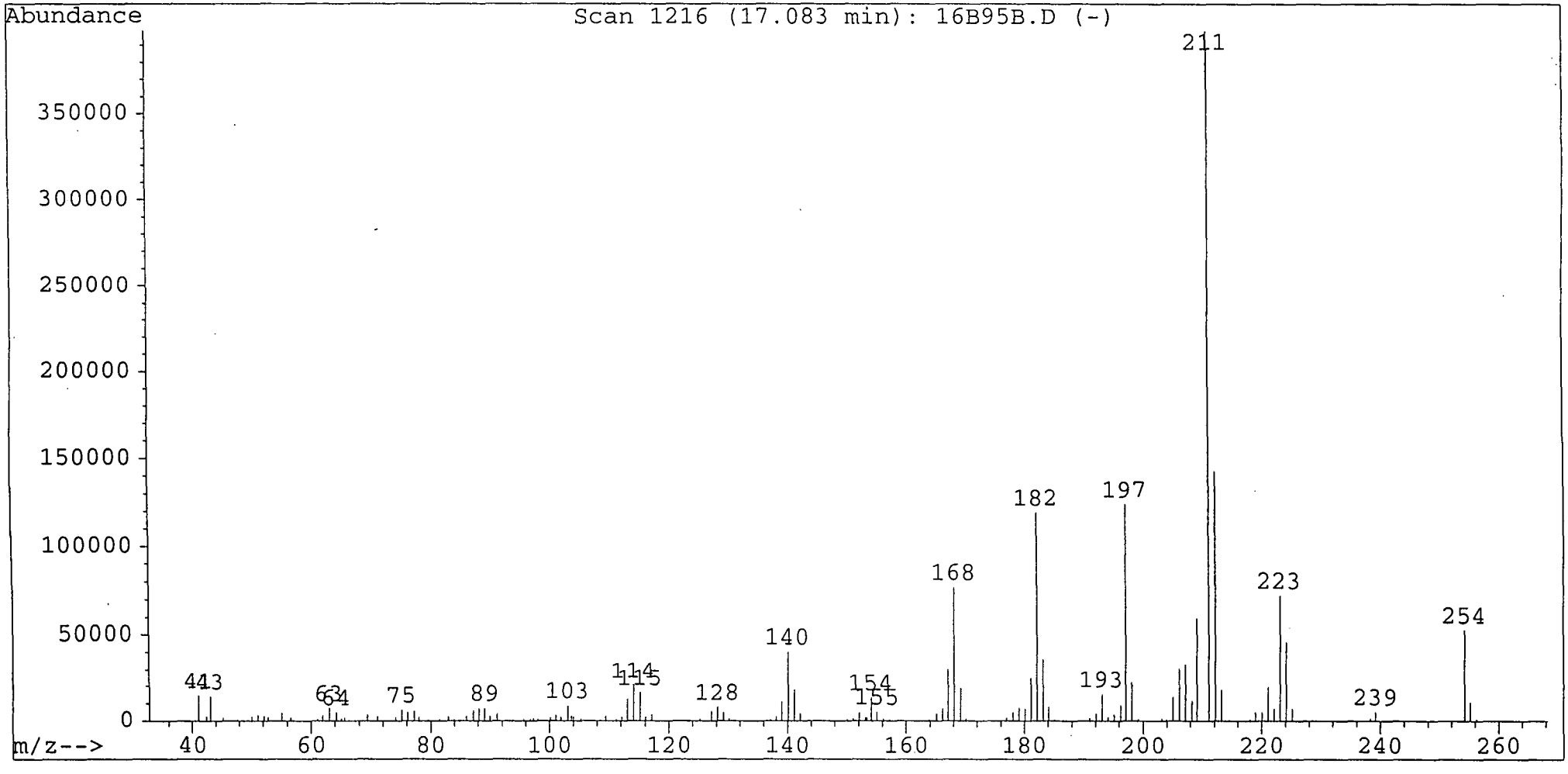


Figure A5.4.4.5.1 GCMS spectrum of compound no. 4 (MW 254).

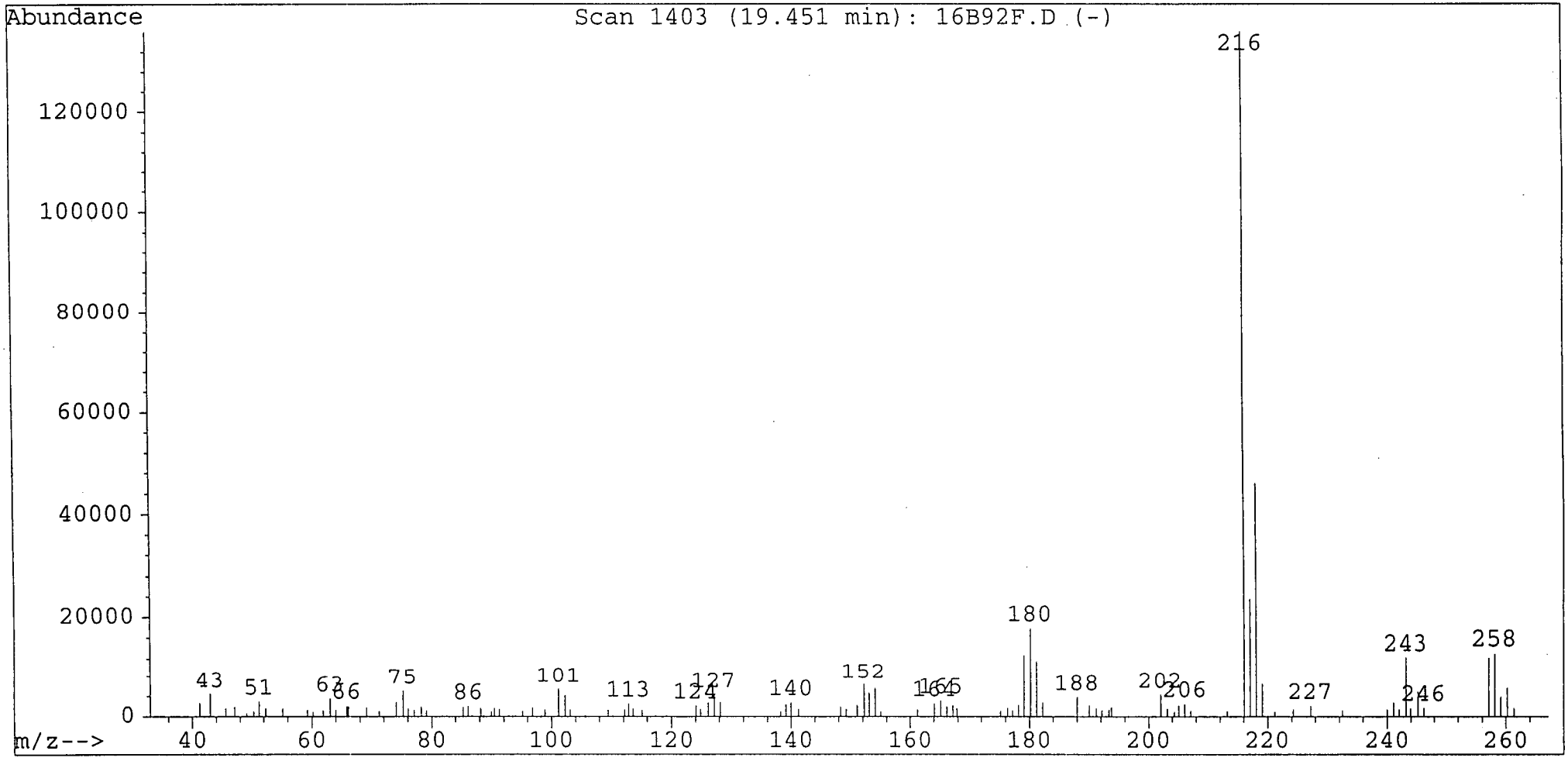


Figure A5.4.4.6.1 GCMS spectrum of compound no. 5 (MW 258).

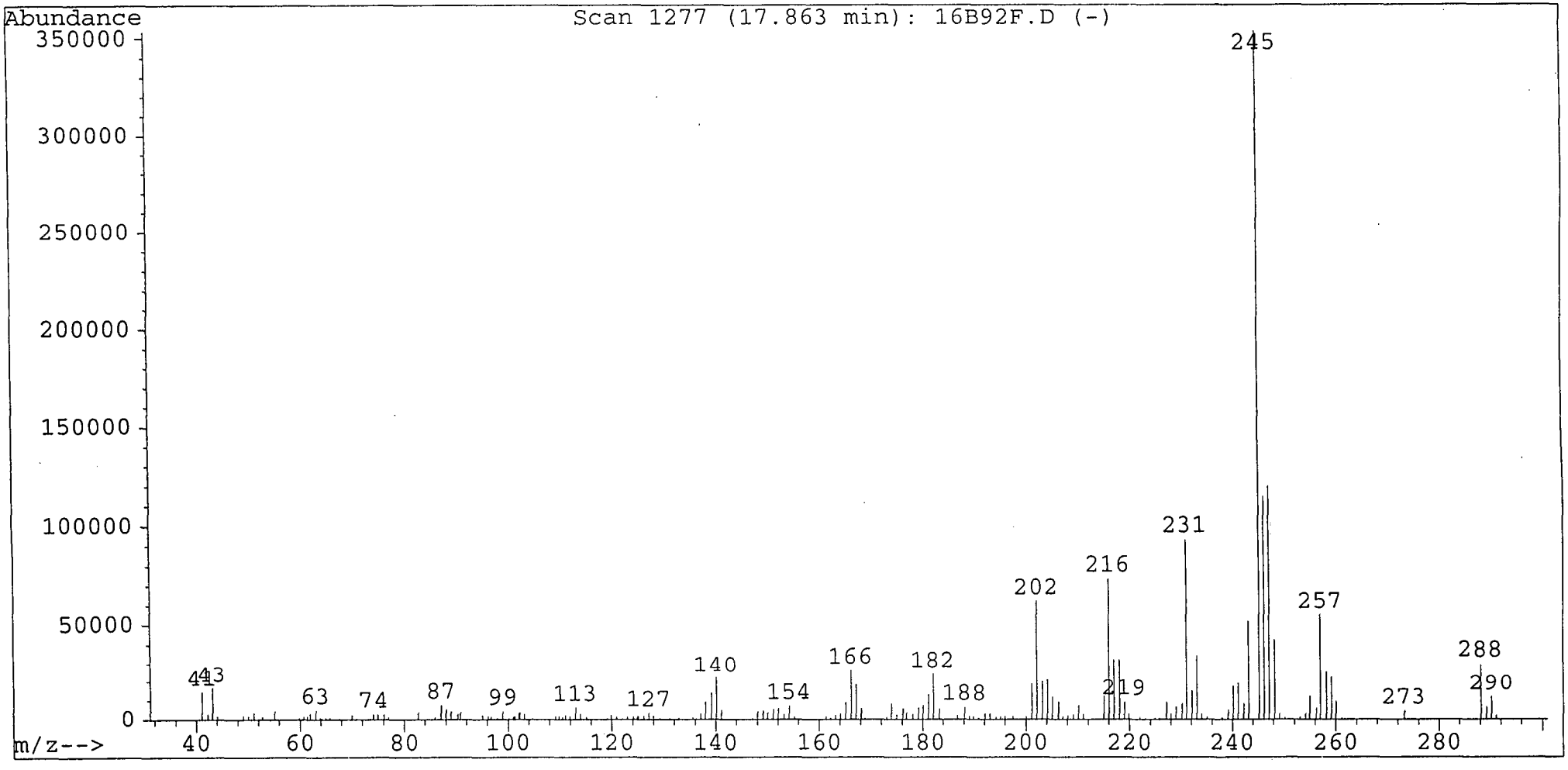


Figure A5.4.4.7.1 GCMS spectrum of compound no. 6 (MW 288).

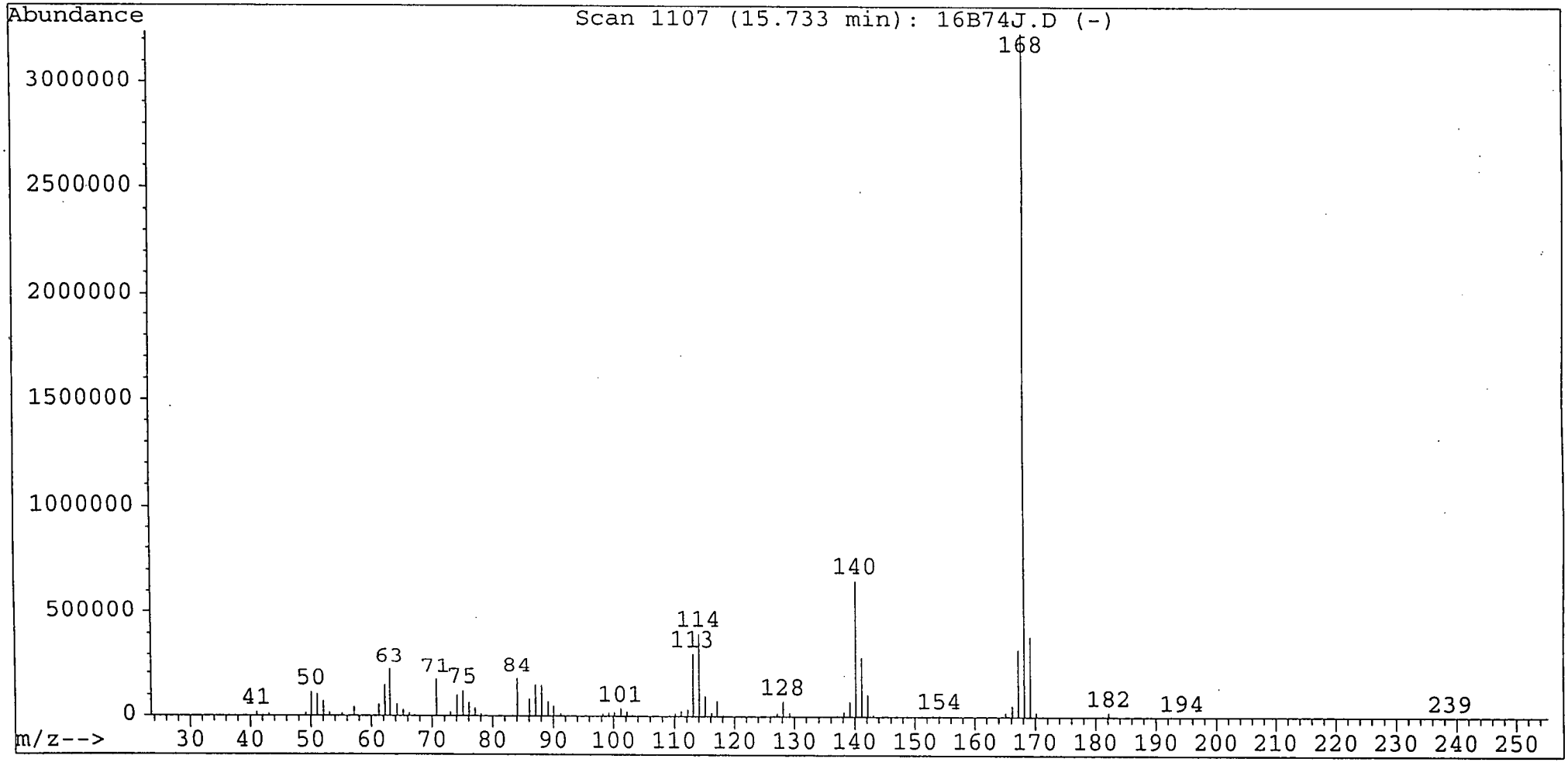


Figure A5.4.5.1.1 GCMS spectrum of norharman (5.4.5.1.1).

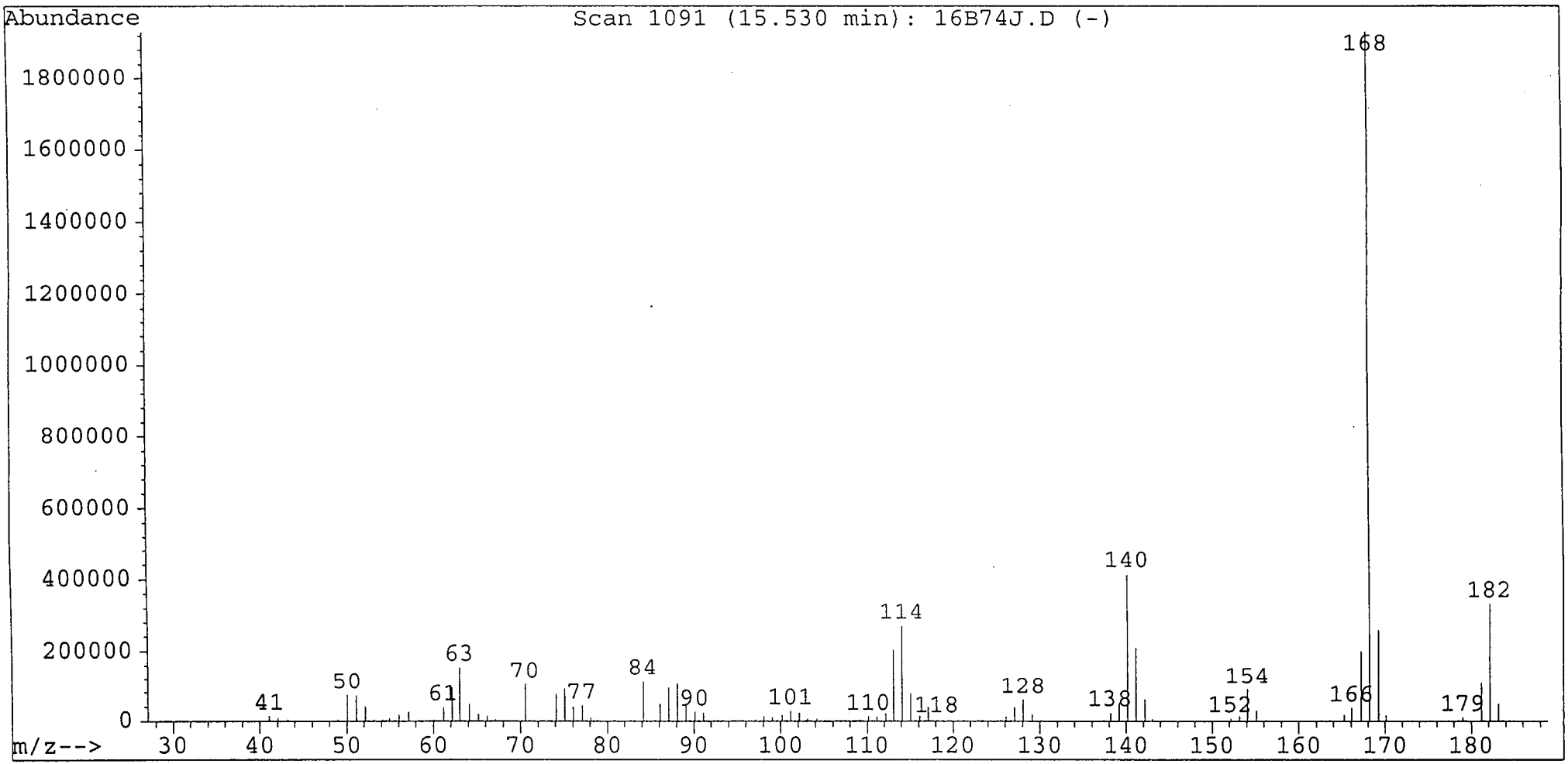


Figure A5.4.5.3.1 GCMS spectrum of compound no. 7 (MW182).

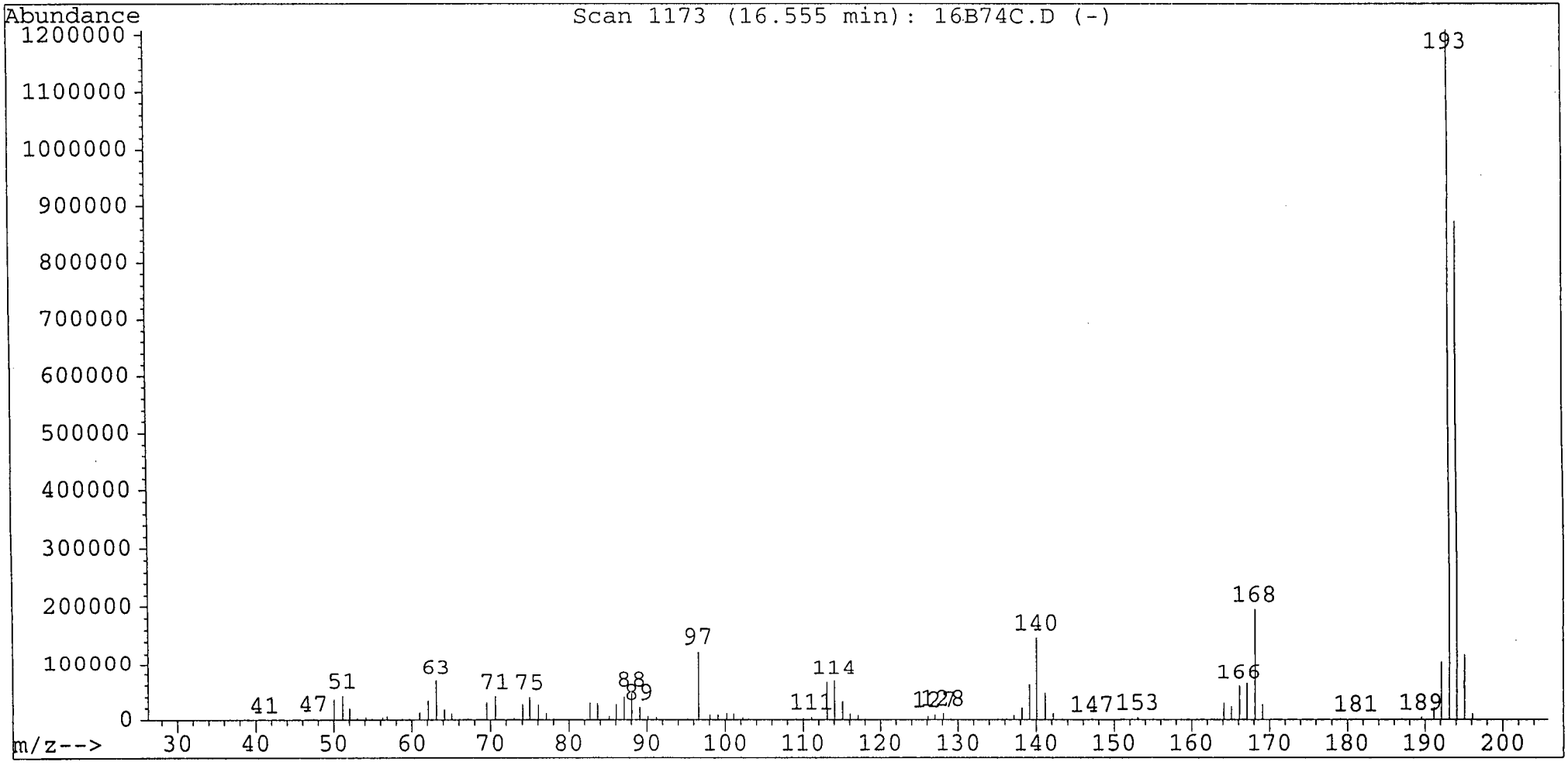


Figure A5.4.5.4.1 GCMS spectrum of pavettine (5.1.3.4).

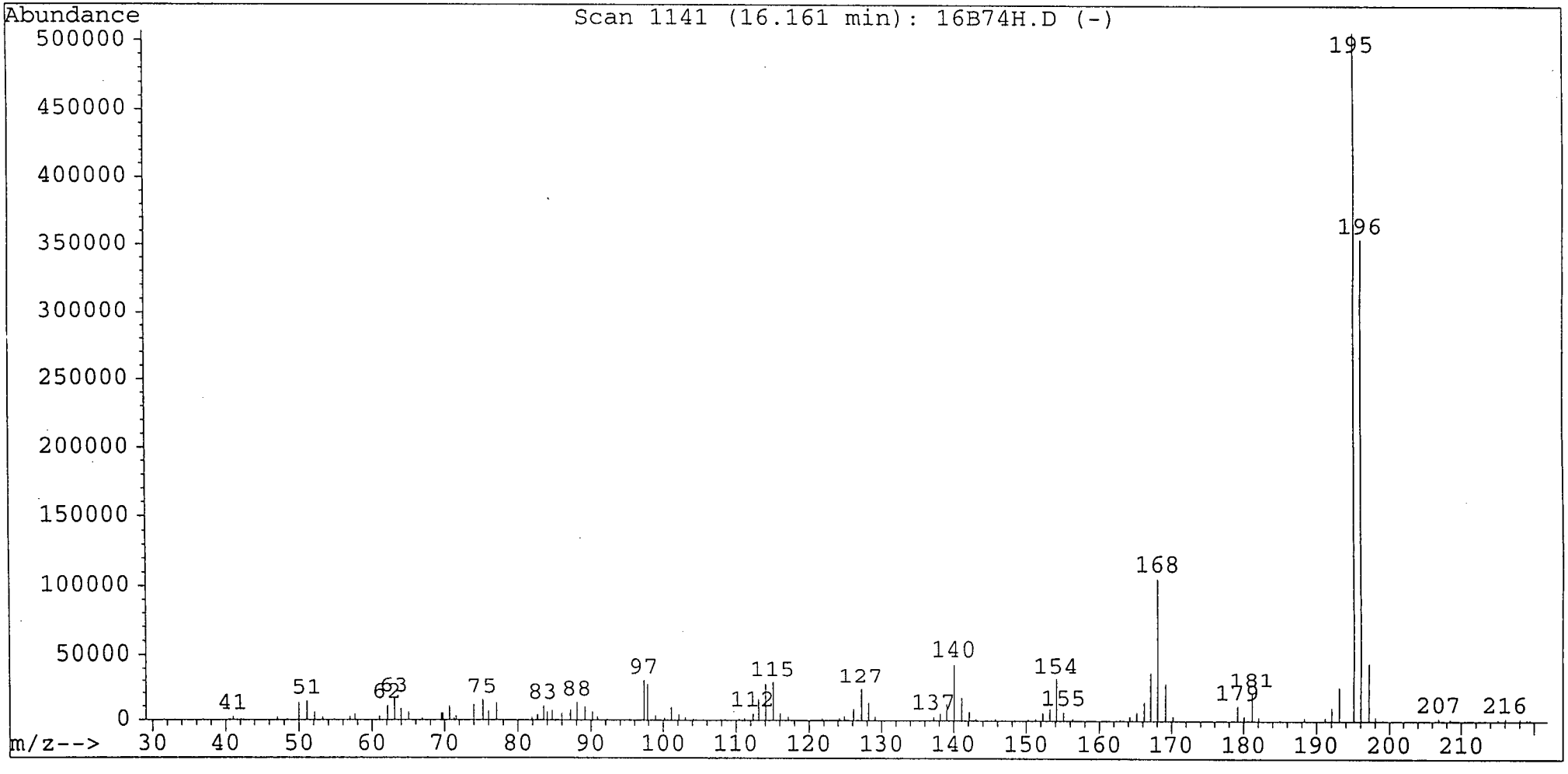


Figure A5.4.5.5.1 GCMS spectrum of 1-ethyl-β-carboline (5.1.3.2).

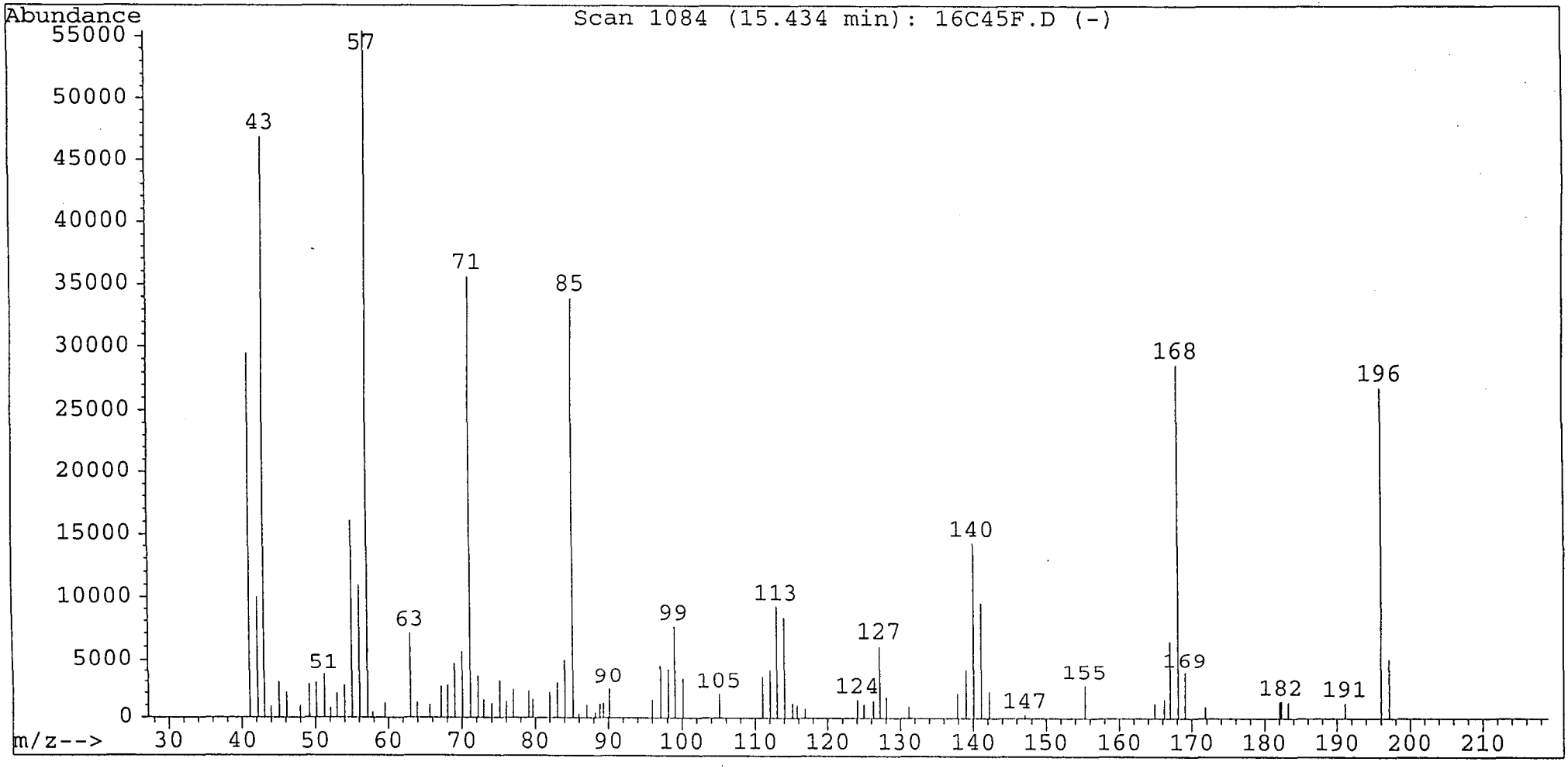


Figure A5.4.5.6.1 GCMS spectrum of compound no. 8 (MW 196).

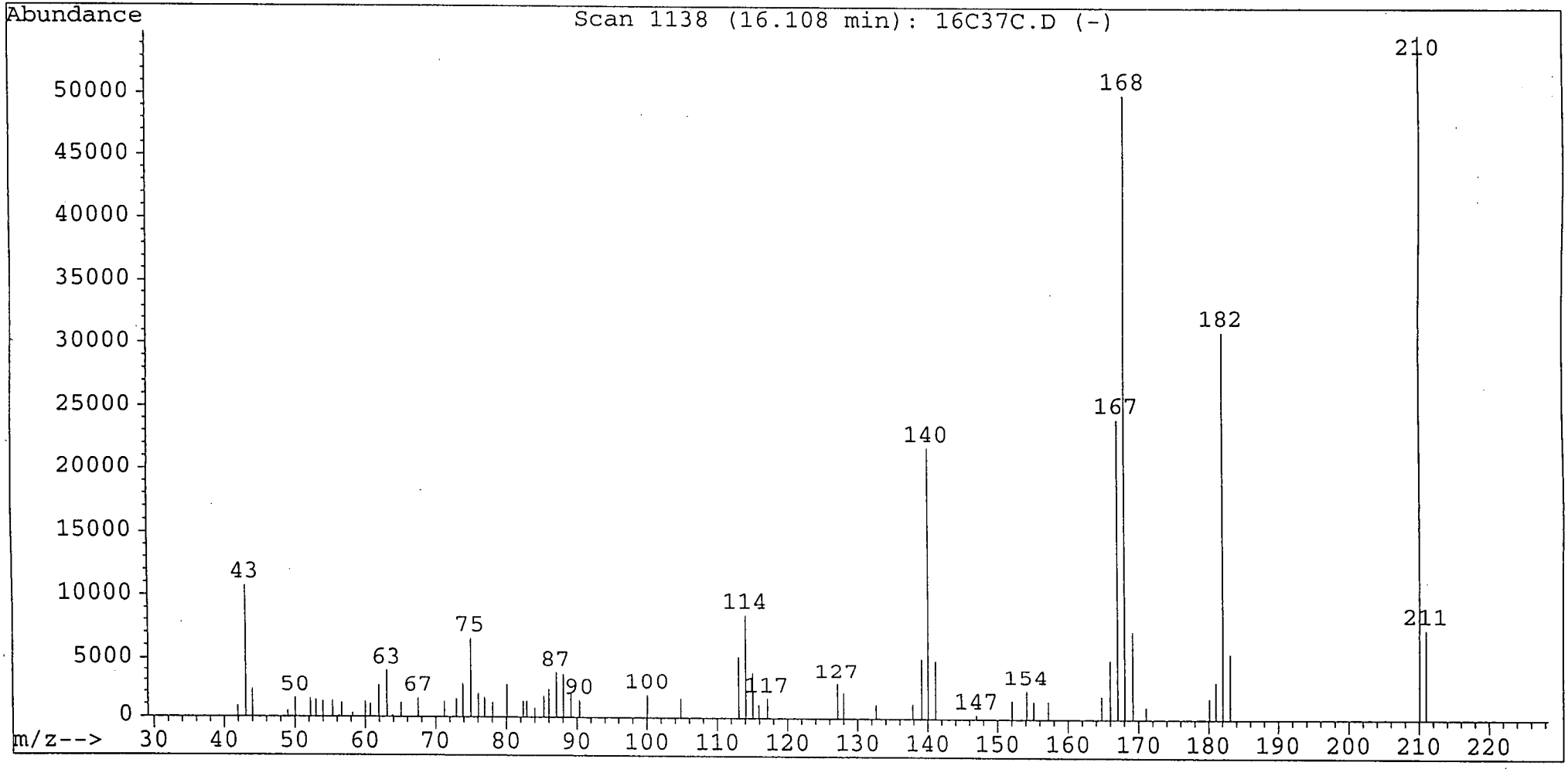


Figure A5.4.5.7.1 GCMS spectrum of 1-acetyl-β-carboline (5.4.4.3.1).

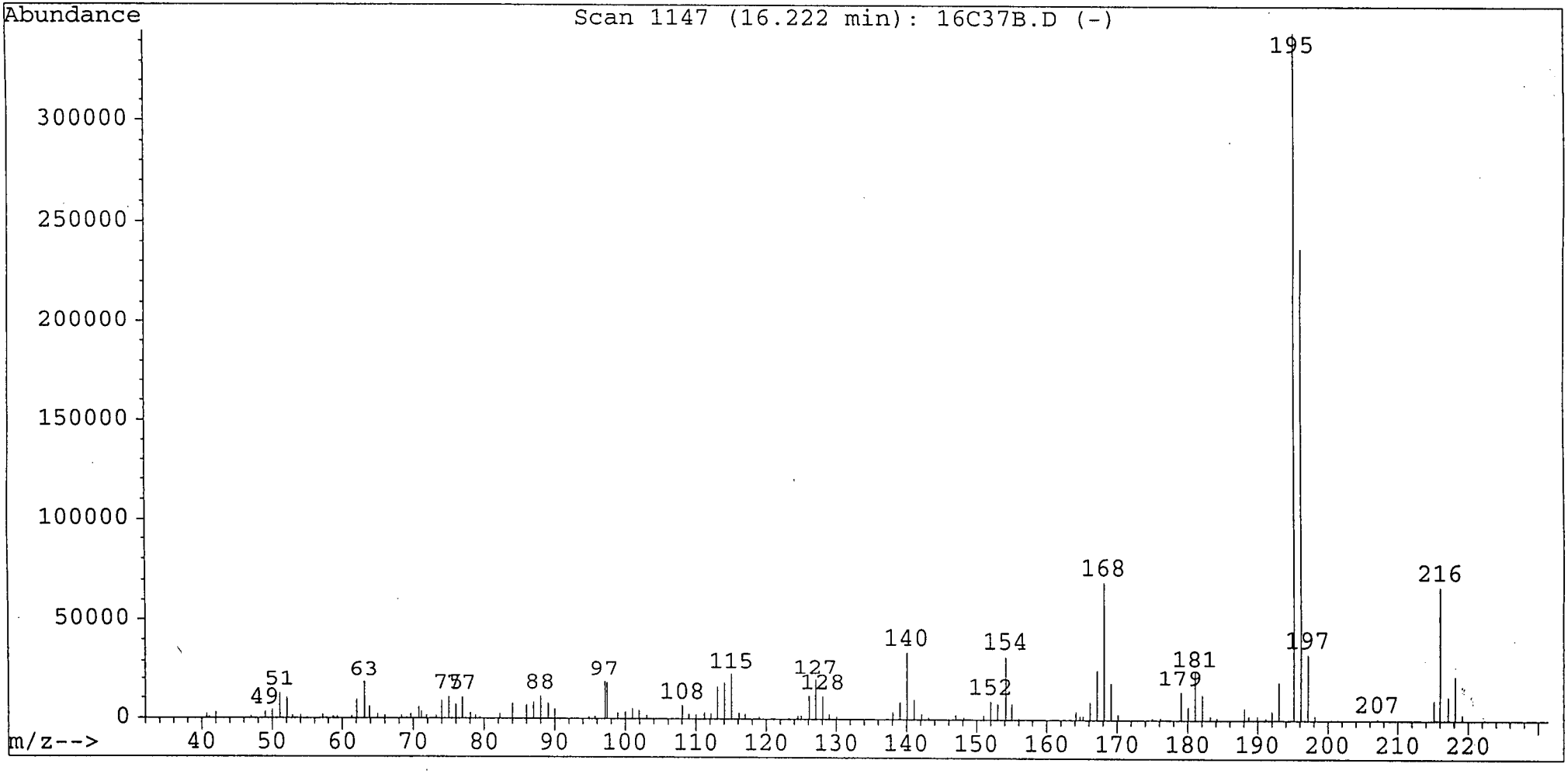


Figure A5.4.5.8.1 GCMS spectrum of compound no. 9 (MW 216).

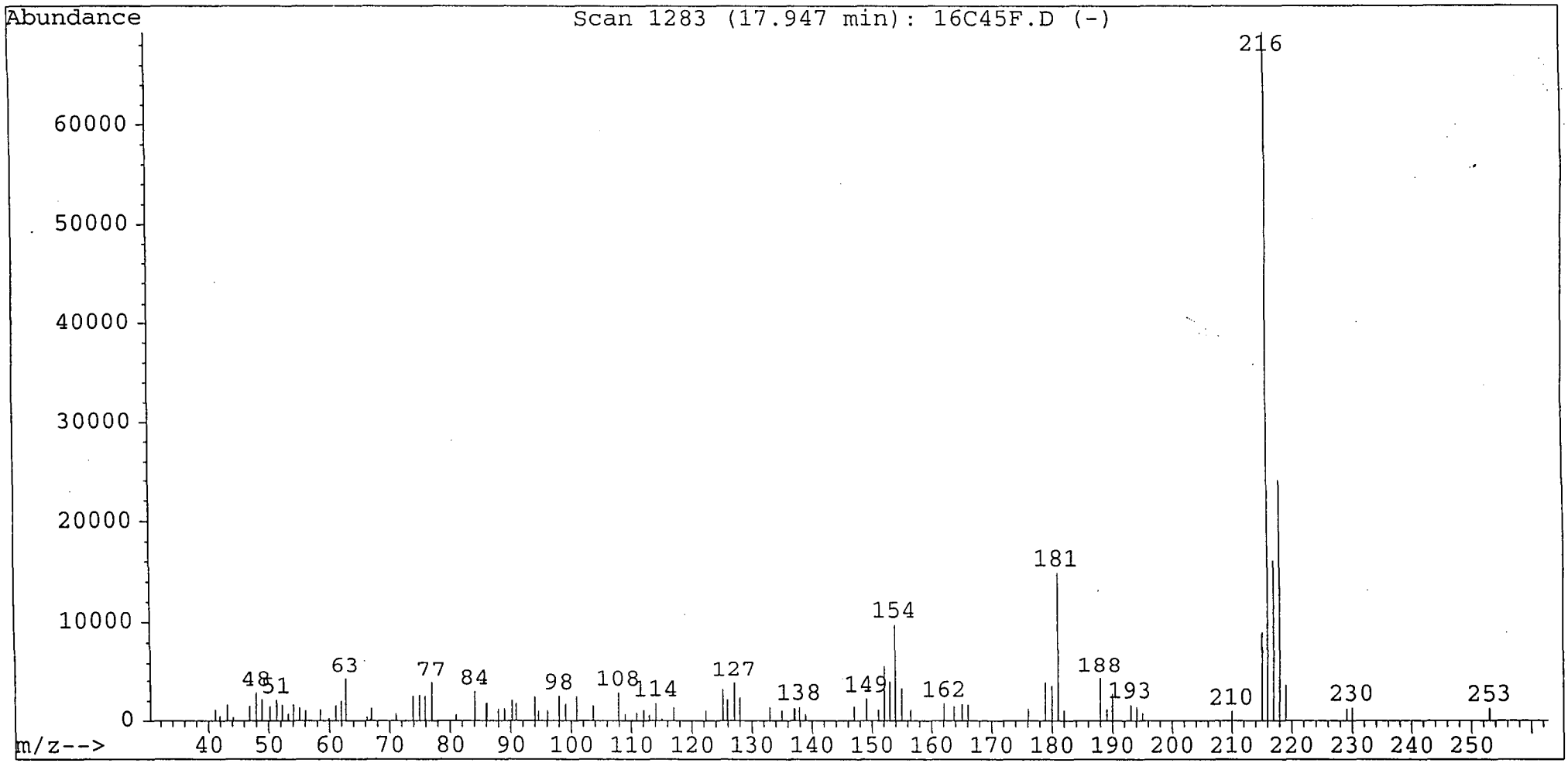


Figure A5.4.5.9.1 GCMS spectrum of compound no.10 (MW 216).

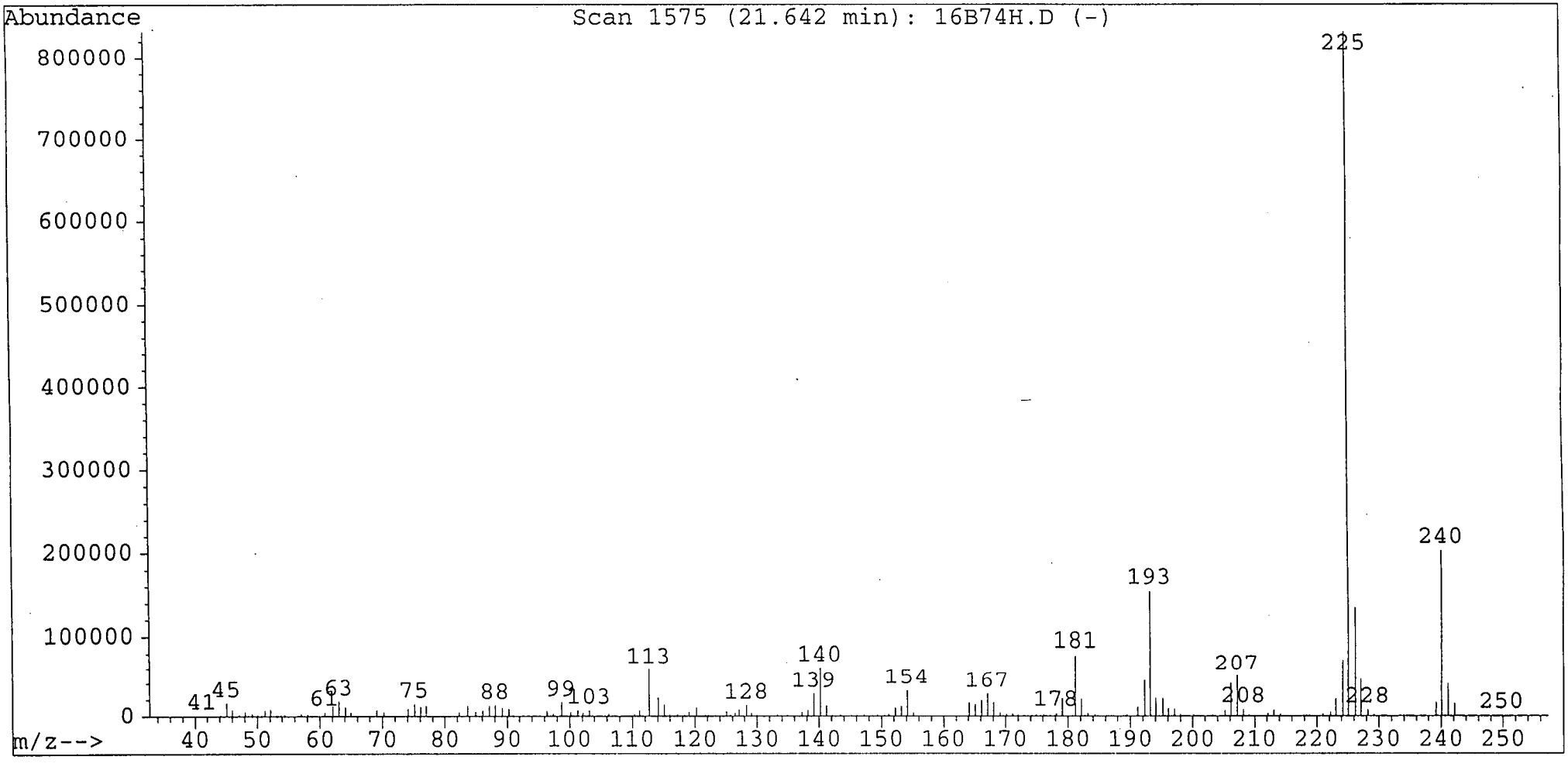


Figure A5.4.5.10.1 GCMS spectrum of compound no. 11 (MW 240).

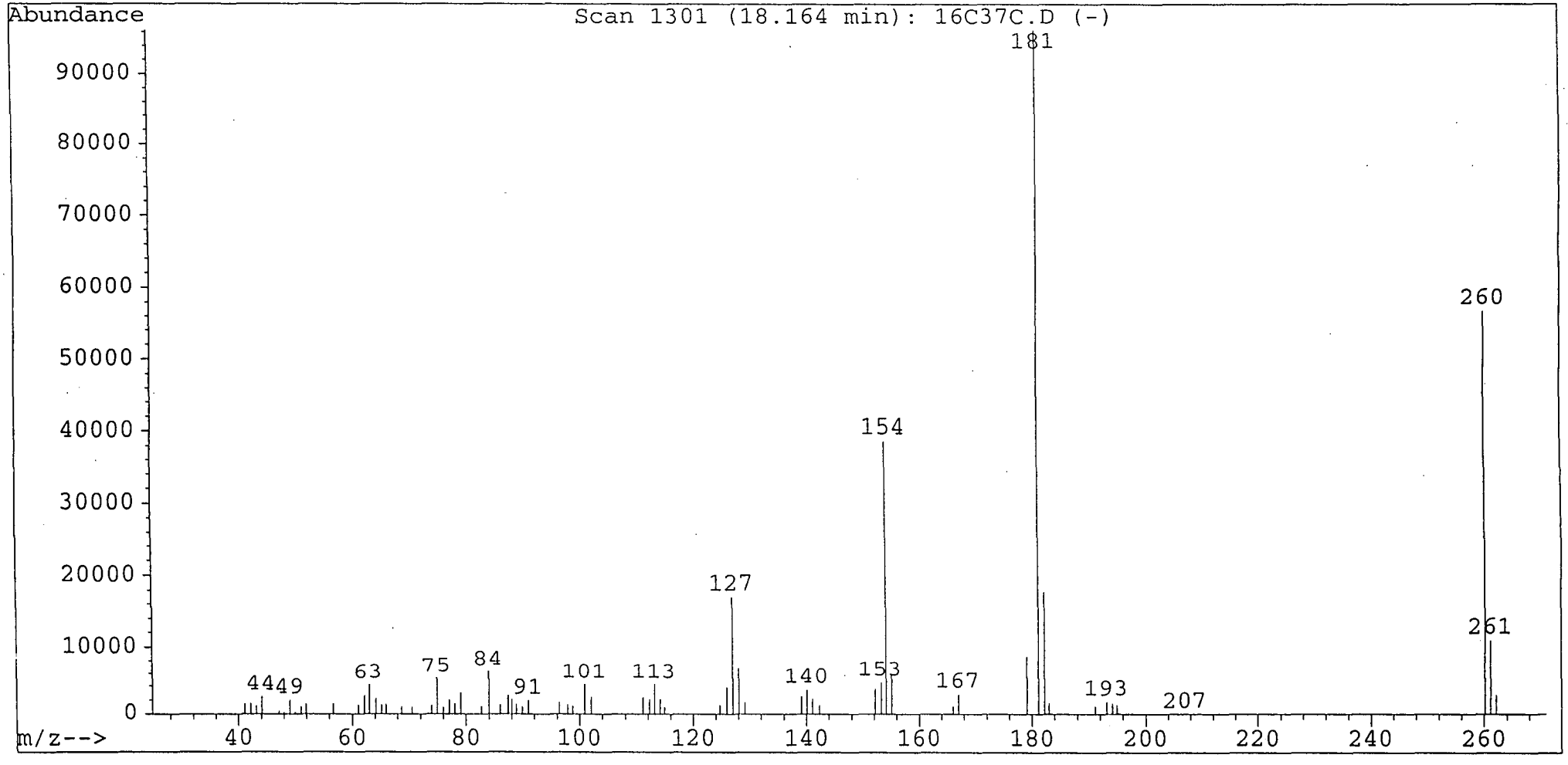


Figure A5.4.5.11.1 GCMS spectrum of compound no. 12 (MW 260).

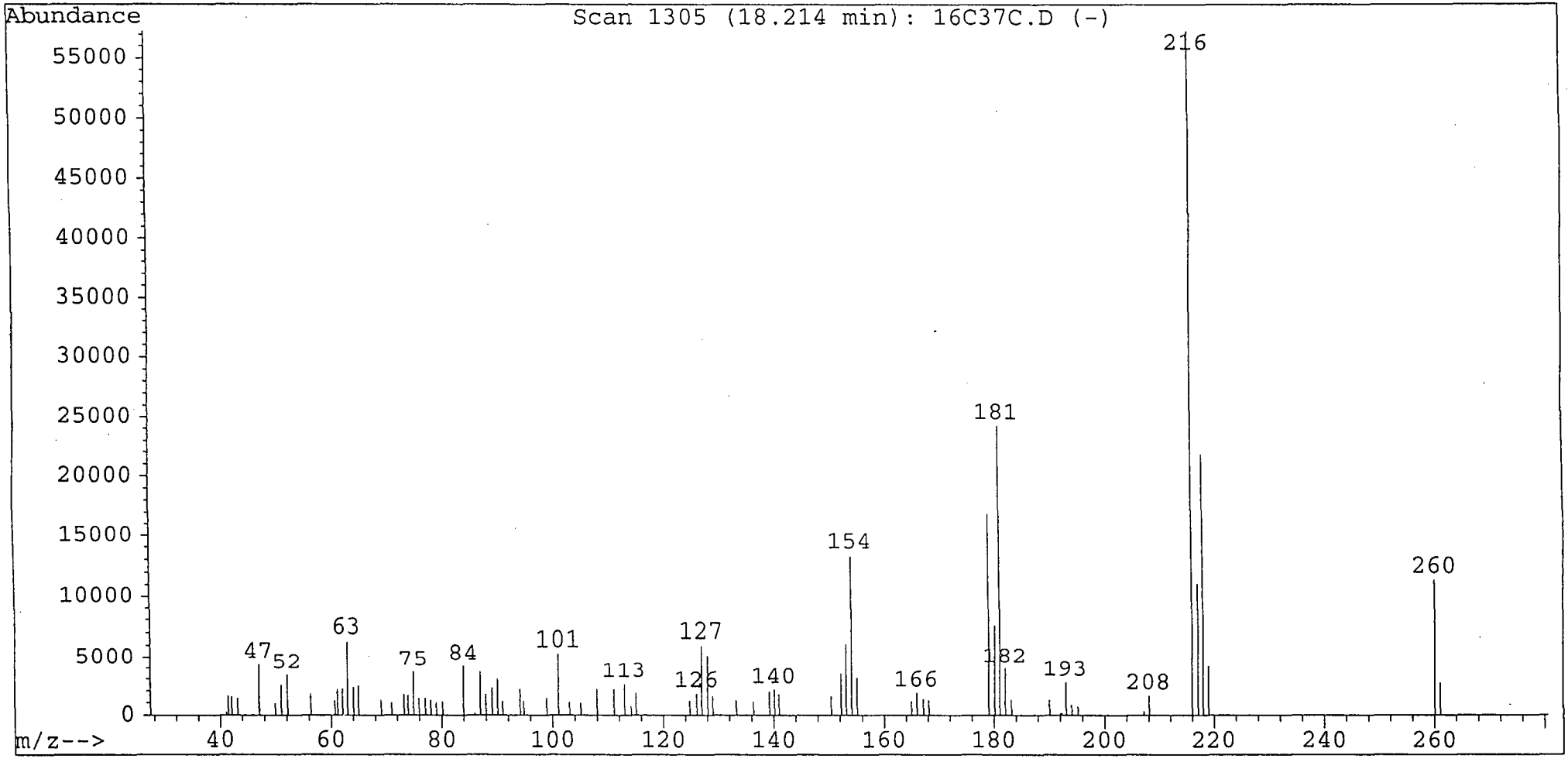


Figure A5.4.5.12.1 GCMS spectrum of compound no. 13 (MW 260).

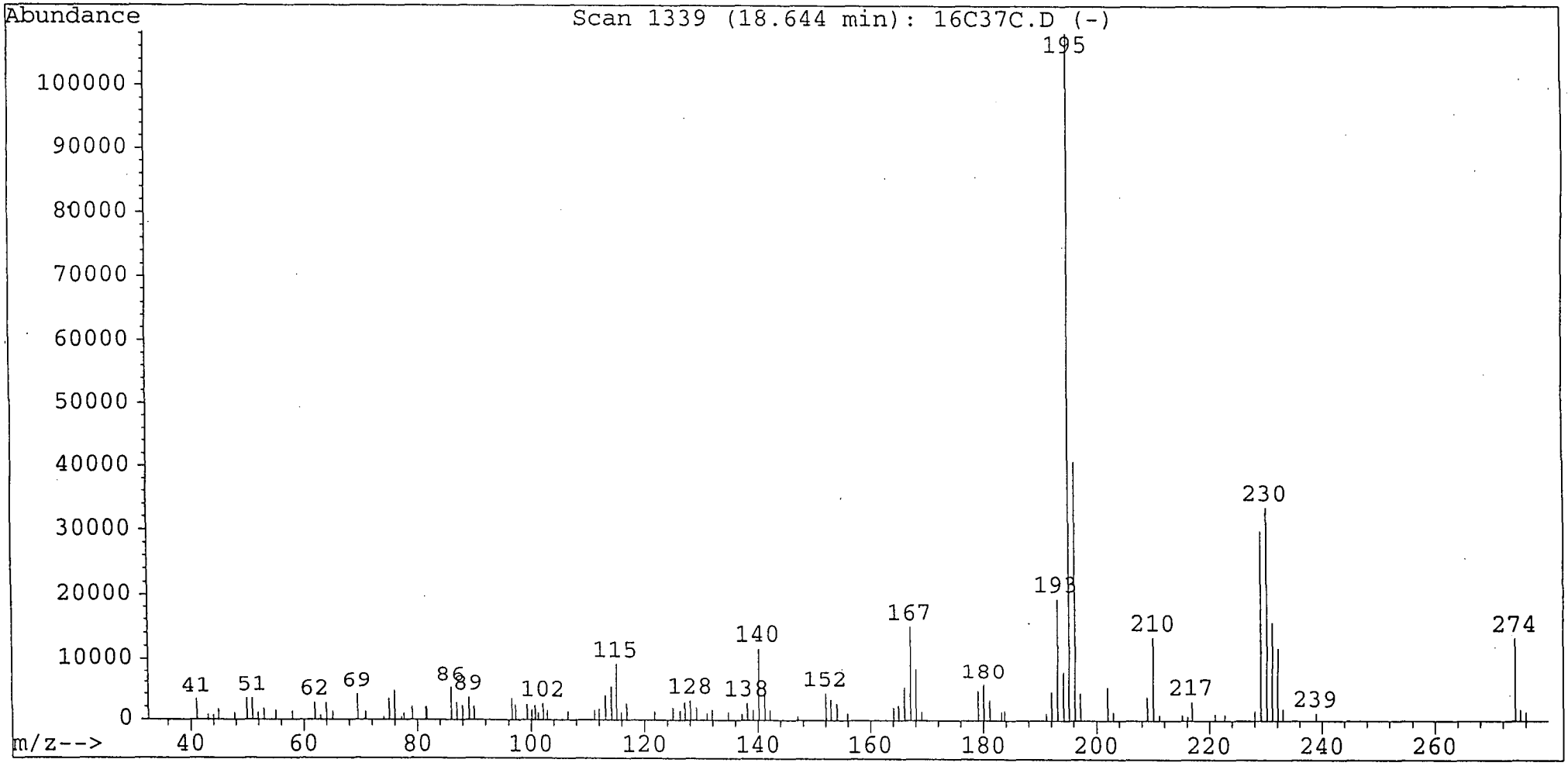


Figure A5.4.5.13.1 GCMS spectrum of compound no. 14 (MW 274).

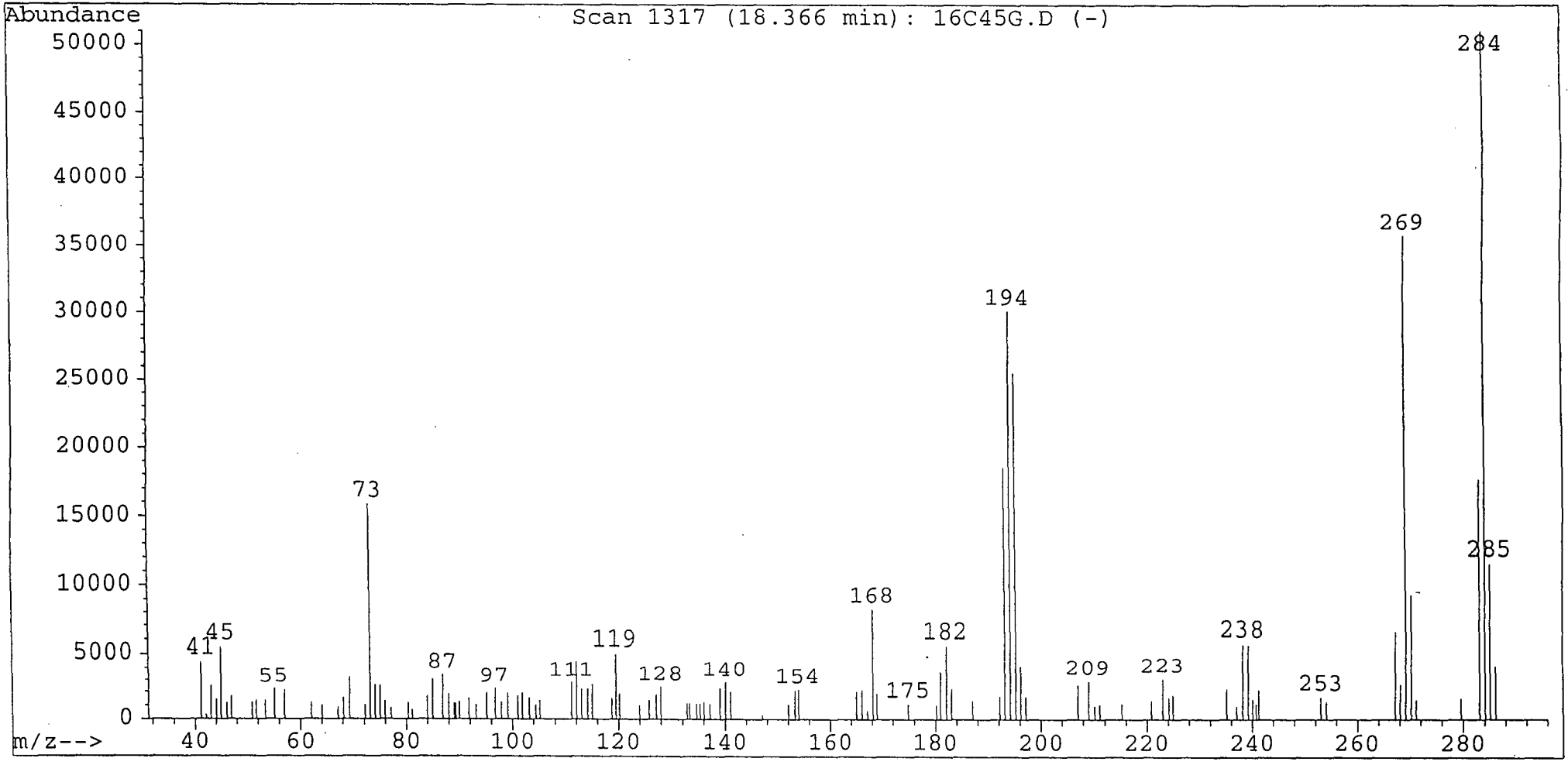


Figure A5.4.5.14.1 GCMS spectrum of compound no. 15 (MW 284).

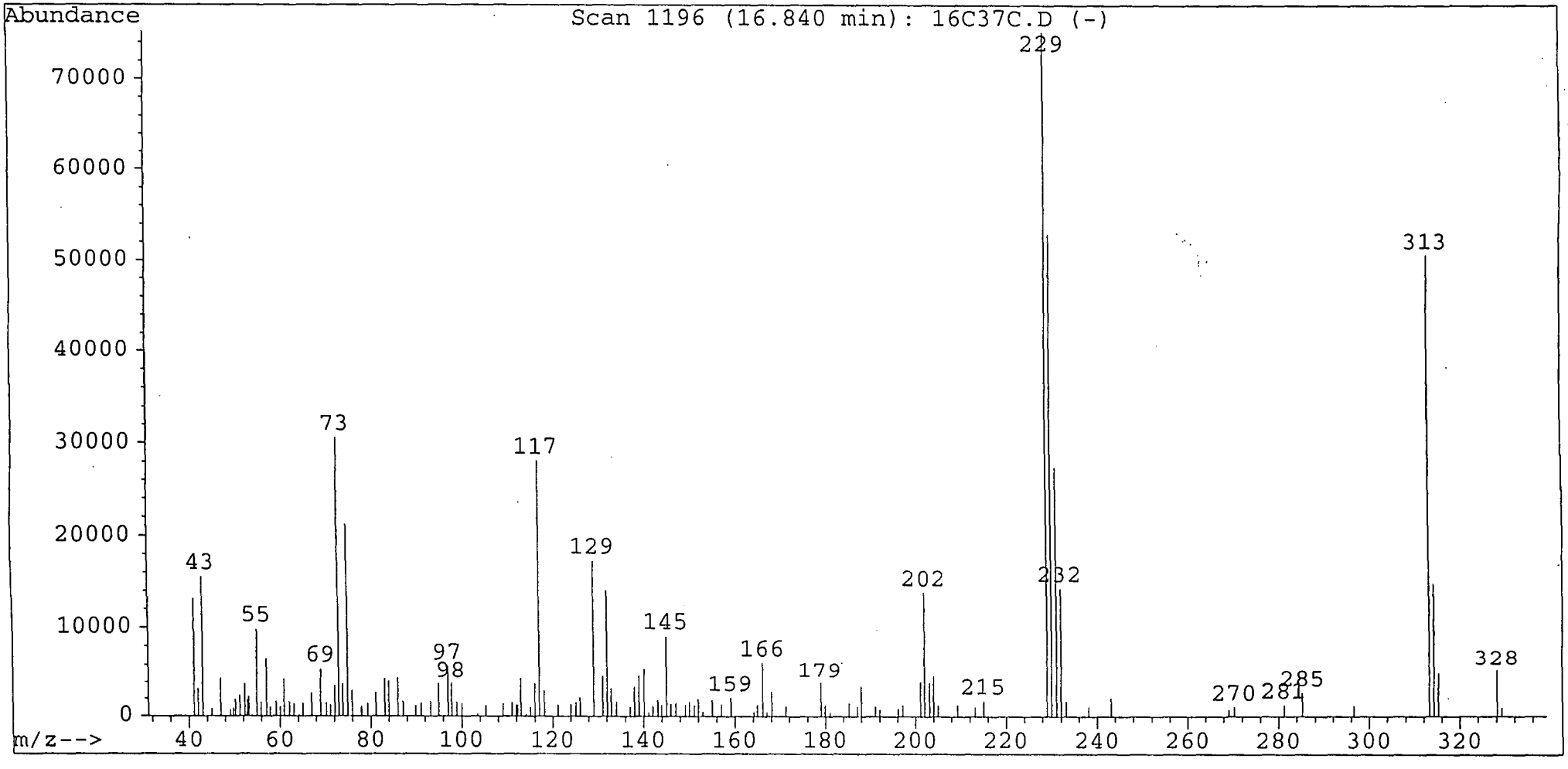


Figure A5.4.5.15.1 GCMS spectrum of compound no. 16 (MW 328).

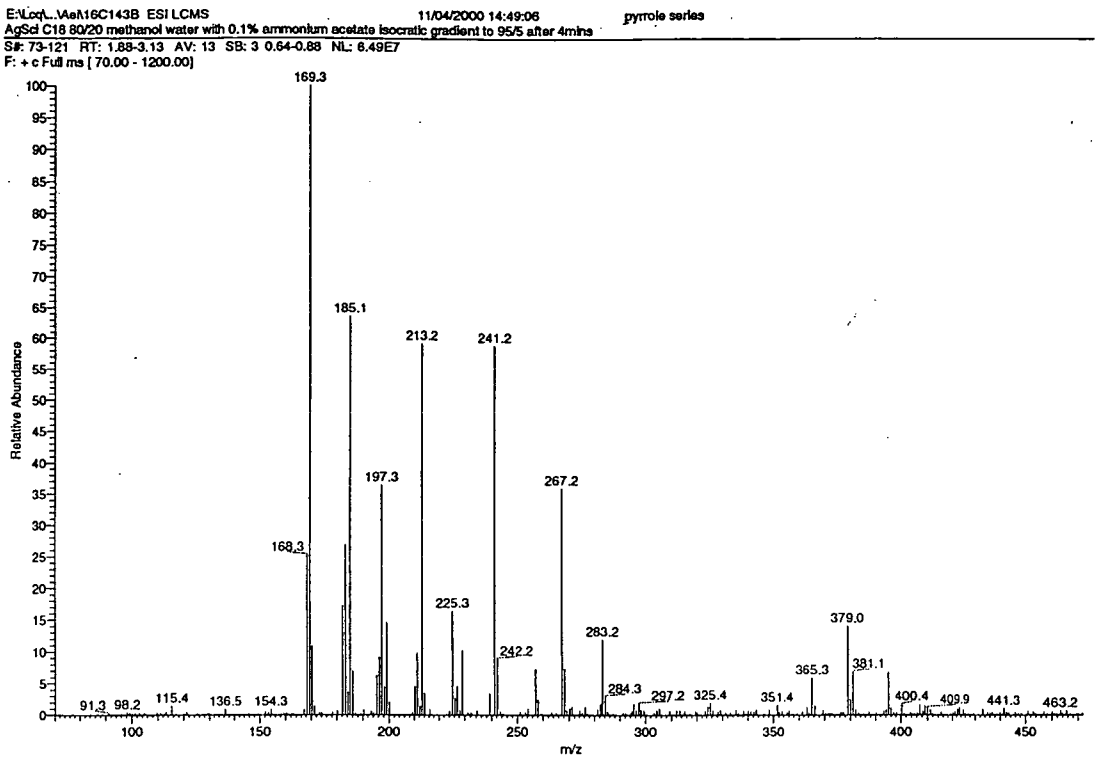


Figure A5.4.5.16.1 ESI LCMS spectrum of the second fraction from the pycnogonid.

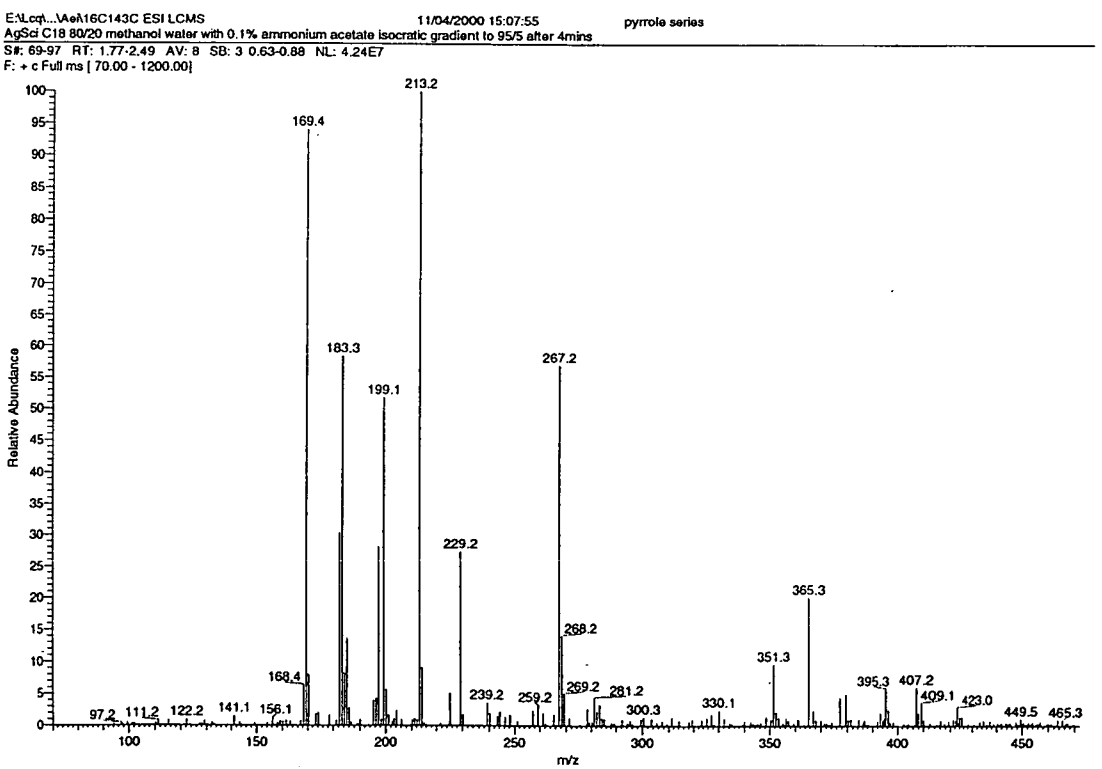


Figure A5.4.5.16.2 ESI LCMS spectrum of the third fraction from the pycnogonid.

Appendix B

List of publications arising from this study:

1. Jongaramruong, J.; Blackman, A. J. *J. Nat. Prod.* **2000**, *63*, 272-275.
2. Jongaramruong, J.; Backman, A. J.; Skelton, B. W.; White, A. H. *Aust. J. Chem.* **2002**, *55*, 275-280.